Entwicklung eines Testsystems zur Erfassung der *in situ* Biodegradation im Grundwasser

D i s s e r t a t i o n

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Abkürzungsverzeichnis

ALA	Altlastenausschuss
B [%]	prozentuale Biodegradation
BACTRAP®	Bakterienfalle (*BACterial TRAP*), Testsystem zum Nachweis der *in situ* Biodegradation
BAME	bakterielle Fettsäuremethylester (*bacterial acid methyl ester*)
BBodSchG	Bundes-Bodenschutzgesetz
BGR	Bundesanstalt für Geowissenschaften und Rohstoffe
BMBF	Bundesministerium für Bildung und Forschung
BTEX	Benzol, Toluol, Ethylbenzol, Xylole
C_0, C_t	Konzentration einer Verbindung zu Beginn (t = 0) oder zu einer bestimmten Zeit (t) der mikrobiellen Umsetzung
C_B	an einer Messstelle zu erwartende Schadstoffkonzentration, die durch mikrobiellen Abbau beeinflusst wurde
$C_{(x)}$	Konzentration einer Verbindung (x)
C_n	Anzahl der Kohlenstoffatome einer Fettsäure
$C_{Schadstoff}$	mineralisierte Schadstofffraktion
CB	Chlorbenzole
CKW	chlorierte Kohlenwasserstoffe
CSIA	Substratspezifische Isotopenfraktionierungsanalyse (*compound-specific stable isotope analysis*)
DBU	Deutsche Bundesstiftung Umwelt
DCB	Dichlorbenzol
DECHEMA	Gesellschaft für Chemische Technik und Biotechnologie
DEHP	Diethylhexylphthalat
DEV	Deutsches Einheitsverfahren
DGGE	denaturierende Gradientengelelektrophorese
DIC	anorganischer, gelöster Kohlenstoff (*dissolved inorganic carbon*)
DIN	Deutsches Institut für Normung
DNA	Desoxyribonukleinsäure (*deoxyribonucleic acid*)
DNAPL	Phasen mit einer Dichte > 1 (*dense non aquous phase liquid*)
DOC	gelöster, organischer Kohlenstoff (*dissolved organic carbon*)
ENA	Stimulation der natürlichen Selbstreinigung (*Enhanced Natural Attenuation*)
Eq.	Gleichung (*equation*)
FA	Fettsäure (*fatty acid*)
FAME	Fettsäuremethylester (*fatty acid methyl ester*)
FD	Filmdicke der GC-Säule

FISH	Fluoreszenz-*in-situ*-Hybridisierung
GC-C-IRMS	Gaschromatographie-Verbrennungs-Isotopenverhältnis-Massenspektroskopie (*Gas chromatography-combustion-isotope ratio mass spectrometry*)
GC-FID	Gaschromatographie-Flammenionisationsdetektor
GC-IRMS	Gaschromatographie-Isotopenverhältnis-Massenspektroskopie (*Gas chromatography-isotope ratio mass spectrometry*)
GC-MS	Gaschromatographie-Massenspektroskopie
Gl.	Gleichung
GSF	Helmholtz Zentrum München, Deutsches Forschungszentrum für Umwelt und Gesundheit
GW	Grundwasser
GWL	Grundwasserleiter
HCH	Hexachlorcyclohexan (Lindan)
IAEA	Internationale Atomenergieorganisation (*International Atomic Energy Agency*)
ID	Innendurchmesser
ITVA	Ingenieurtechnischer Verband Altlasten
IMW	Innovative Messtechnik Weiss
KORA	Kontrollierter natürlicher Rückhalt und Abbau von Schadstoffen bei der Sanierung kontaminierter Böden und Grundwässer
LABO	Bund-/Länderarbeitsgemeinschaft Bodenschutz
LHKW	leichtflüchtige, halogenierte Kohlenwasserstoffe
MCB	Monochlorbenzol
MKW	Mineralölkohlenwasserstoffe
MNA	Überwachung der natürlichen Selbstreinigung (*Monitored Natural Attenuation*)
MLPS	Multilevel-Packersystem
m uGOK	Meter unter Geländeoberkante
MPN	Wahrscheinlichste Keimzahl Methode (Most-Probable-Number-Methode)
MTBE	Methyl-*tert*-butylether
NA	Natürliche Selbstreinigung (*Natural Attenuation*)
n.d.	nicht bestimmt (not determined)
p. A.	Pro analysi (zur Analyse, Reinheitsgrad der Chemikalien)
PAC	Aktivkohle (*powdered activated carbon*)
PAH	polyzyklische aromatische Kohlenwasserstoffe (*polycylic aromatic hydrocarbon*)
PAK	polyzyklische aromatische Kohlenwasserstoffe
PCA	*Principal Component Analysis*
PC1/2	*Principal Component 1/2*
PCE	Tetrachlorethen (Perchlorethen)

PCR	Polymerase-Kettenreaktion (*polymerase chain reaction*)
REACH	Verordnung zur Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe (***R**egistration, **E**valuation, **A**uthorisation of **Ch**emicals*)
PLFA	Phospholipid-Fettsäure (*phospholipid-derived fatty acid*)
RDH	reduktive Dehalogenierung
RNA	Ribonukleinsäure (*ribonucleic acid*)
rRNA	ribosomale Ribonukleinsäure (*ribosomal ribonucleic acid*)
R_0, R_t	Isotopenverhältnis einer Verbindung zu Beginn (t = 0) oder zu einer bestimmten Zeit (t) der mikrobiellen Umsetzung
$R_{(x)}$	Isotopenverhältnis einer Verbindung (x)
SAFIRA	Sanierungsforschung in regional kontaminierten Aquiferen
SI	*Supporting Information*
SIFA	Analyse der Fraktionierung stabiler Isotope (*stable isotope fractionation analysis*)
SIP	Stabile Isotopenbeprobung (*stable isotope probing*)
SSCP	*Single Strand Conformation Polymorphism* (molekulares Nachweisverfahren)
Tab.	Tabelle
TCB	Trichlorbenzol
TEA	terminaler Elektronenakzeptor (*terminal electron acceptor*)
TLFA	*Total Lipid Fatty Acid*
TMCS	Trimethylchlorosilane
TOC	gesamter organischer Kohlenstoff (*total organic carbon*)
UFZ	Helmholtz-Zentrum für Umweltforschung
US-EPA	US-amerikanische Umweltbehörde (United States Environmental Protection Agency)
V-CTD	Vienna Canyon Diabolo Troilite
V-PDB	Vienna PeeDee Belemnite
V-SMOC	Vienna Standard Mean Ocean Chloride
V-SMOW	Vienna Standard Mean Ocean Water
WHO	Weltgesundheitsorganisation (*World Health Organization*)
$y_{x/s}$	Ertragskoeffizient
Z_C	Anzahl der Kohlenstoffatome des Schadstoffmoleküls
α	Isotopenfraktionierungsfaktor
$\delta^{13}C_{(x)}$	Kohlenstoffisotopenverhältnis einer Verbindung (x)
ε	Anreicherungsfaktor
$\varepsilon_{DIC\text{-}CH4}$	Anreicherungsfaktor aus der Differenz zwischen der Kohlenstoffisotopensignatur des DIC und Methans

Kurzfassung

Die natürlichen Abbau- und Rückhalteprozesse (*Natural Attenuation*) gewinnen in der Altlastenpraxis zunehmend an Bedeutung. Insbesondere an großräumig kontaminierten Standorten (*Megasites*), wo Kosten und Nutzen herkömmlicher Sanierungsmaßnahmen oft in keinem angemessenen Verhältnis stehen, wird *Monitored Natural Attenuation* (MNA) immer häufiger zur Überwachung und Sicherung von Altlastenstandorten in Betracht gezogen. Neben den abiotischen Prozessen ist hierbei vor allem die *in situ* Biodegradation von wesentlicher Bedeutung, da der mikrobielle Abbau zu einer nachhaltigen Reduzierung der Schadstoffe im Grundwasser beiträgt und oft die wichtigste Schadstoffsenke darstellt. Die Implementierung von MNA als Standortsicherungskonzept erfordert eine umfangreiche Charakterisierung des natürlichen Selbstreinigungspotenzials von Grundwasserleitern, wobei der Nachweis der mikrobiellen *in situ* Aktivität von zentraler Bedeutung ist.

Zur Erfassung der *in situ* Biodegradation in kontaminierten Aquiferen gibt es eine Vielzahl verschiedener Methoden, wobei der Großteil dieser konventionellen Verfahren nur einen indirekten Nachweis des mikrobiellen Abbaupotenzials ermöglicht. Mit der Entwicklung eines neuen Testsystems, sogenannten *in situ* Mikrokosmen (BACTRAP®), steht neben der substratspezifischen Isotopenanalyse (CSIA) und *in situ* Tracerversuchen ein weiteres Verfahren für den direkten Nachweis des mikrobiellen Schadstoffabbaus zur Verfügung.

Die Aufgaben dieser Promotion bestanden insbesondere in (I) der Validierung des Testsystems zur Beurteilung der *in situ* Biodegradation, (II) der Anwendung von *in situ* Mikrokosmen an verschiedenen Feldstandorten und für unterschiedliche Schadstoffe zur Evaluierung des Verfahrens mithilfe alternativer Methoden, (III) der Untersuchung des intrinsischen Abbaupotenzials persistenter Schadstoffe, (IV) der Weiterentwicklung der Methode zur Analyse der am Schadstoffabbau beteiligten mikrobiellen Konsortien und (V) zur Rekonstruktion von Abbauwegen mithilfe von Metabolitenuntersuchungen. Darüber hinaus stellte die (VI) Implementierung des neuen Testsystems in die Altlastenpraxis zum Nachweis der *in situ* Biodegradation im Kontext von *Natural Attenuation* einen weiteren Schwerpunkt dieser Arbeit dar.

Durch den Einsatz ^{13}C-markierter Substrate als Tracer in *in situ* Mikrokosmen konnte die Biodegradation persistenter Verbindungen direkt im kontaminierten Aquifer innerhalb weniger Monate nachgewiesen werden. Das Testsystem wurde im Rahmen dieser Promotion erfolgreich an verschiedenen Standorten zum Nachweis des anaeroben Abbaus von Benzol, Toluol, Monochlorbenzol (MCB) sowie verschiedener polyzyklischer aromatischer Kohlenwasserstoffe (PAK) eingesetzt. Ein Großteil der Feldversuche erfolgte in enger Zusammenarbeit mit Kooperationspartnern aus der Industrie.

Für die Charakterisierung des anaeroben Benzolabbaus sowie zur Validierung der *in situ* Mikrokosmen wurde am Standort eines ehemaligen Hydrierwerks die vertikale Struktur einer

Benzolfahne im Rahmen eines Multilevel-Experimentes mithilfe geochemischer und isotopenchemischer Methoden untersucht (Kapitel 2). Die einzelnen Ergebnisse der verschiedenen Methoden zeigten generell gute Übereinstimmung hinsichtlich einer Abschätzung des *in situ* Abbaupotenzials. Im hoch kontaminierten Bereich der Fahne konnte der Benzolabbau mit der Methode der Isotopenfraktionierung jedoch nicht nachgewiesen werden. Nur der Einsatz ^{13}C-markierter Substrate in *in situ* Mikrokosmen ermöglichte einen sensitiven Nachweis der Biodegradation in allen Zonen der Fahne.

Unter zu Hilfenahme von DNA Fingerprinting Methoden konnte gezeigt werden, dass die mikrobielle Gemeinschaft auf den *in situ* Mikrokosmen im Wesentlichen der Grundwassermikroflora des BTEX kontaminierten Aquifers entspricht (Kapitel 3, 4). Weitere Feldversuche zur Charakterisierung der Benzol- und Toluol abbauenden Mikroorganismen verdeutlichten, dass *in situ* Mikrokosmen geeignet sind, um in Kombination mit Nukleinsäure basierenden molekularbiologischen Methoden empfindlich die mikrobielle Gemeinschaft in Übereinstimmung mit der regionalen Geochemie des Aquifers abzubilden. Die Analyse der Fettsäuremuster hingegen ist für taxonomische Fragestellungen ungeeignet.

Ein weiterer Indikator für *in situ* Abbauaktivität ist der Nachweis charakteristischer ^{13}C-markierter Metabolite, anhand derer Aussagen bezüglich der Abbaumechanismen möglich sind. In diesem Zusammenhang konnte Benzylsuccinat, als typischer Metabolit des anaeroben Toluolabbaus (Kapitel 3) sowie Benzol als Intermediat der reduktiven Dehalogenierung von MCB (Kapitel 7) von *in situ* Mikrokosmen extrahiert werden. Durch die Verwendung sorptiver Trägermaterialien wie Aktivkohle können sich Metabolite auf dem *in situ* Testsystem anreichern. Die Analyse des Metabolitenspektrums bietet daher das Potenzial, neben dem Nachweis des Schadstoffabbaus auch bisher noch unbekannte Abbauwege zu erforschen.

Mithilfe von Isotopenmethoden konnte unter anoxischen Bedingungen erstmals die vollständige Mineralisierung von MCB in Laborabbauversuchen nachgewiesen werden (Kapitel 5, 6). Des Weiteren ermöglichten *in situ* Mikrokosmen den Nachweis des anaeroben MCB Abbaus unter *in situ* Bedingungen an verschiedenen Standorten (Kapitel 5-7). MCB diente dabei als Modellsubstanz für schwer abbaubare Verbindungen. Die Untersuchungen am Standort Bitterfeld erfolgten in einem Multilevel-Experiment (Kapitel 5) sowie in einer Pflanzenkläranlage, wobei hier die *in situ* Mikrokosmen erstmals direkt im Sedimentkörper inkubiert wurden (Kapitel 7). Daraus lässt sich ableiten, dass das BACTRAP-Testsystem auch direkt in Böden und Sedimenten eingesetzt werden kann.

Der direkte Nachweis des *in situ* Abbaus einzelner Schadstoffe ist mithilfe stabiler Isotopentechniken wie CSIA und *in situ* Mikrokosmen auch an Standorten mit komplexem Kontaminationsmuster möglich. Für die Charakterisierung des *Natural Attenuation* Potenzials an einem Chlorbenzol kontaminierten Standort wurde hierfür ein integratives Untersuchungsverfahren entwickelt (Kapitel 6). Da die Anwendung des Rayleigh Konzepts zur

Abschätzung der *in situ* Biodegradation anhand der Änderung der Isotopensignaturen für sequenzielle Abbauwege wie die reduktive Dehalogenierung limitiert ist, wurde eine Isotopenbilanz erstellt. Neben den innovativen Isotopentechniken diente die statistische Auswertung umfangreicher Datensätze zur Charakterisierung der heterogenen hydrogeochemischen Bedingungen. Trotz der komplexen Standortbedingungen konnten mithilfe dieses integrativen Ansatzes entsprechend bestehender nationaler und internationaler Richtlinien (Hessisches Landesamt für Umwelt und Geologie, 2004; ITVA, 2004; KORA, 2007; US-EPA, 1999) mehrere Beweislinien für den Nachweis der *in situ* Abbauprozesse erbracht werden.

Mit dieser Arbeit wurde gezeigt, dass *in situ* Mikrokosmen geeignet sind, um die Aktivität, Struktur und Funktion von am Schadstoffabbau beteiligten Mikroorganismen unter Einsatz ^{13}C-markierter Tracerverbindungen mit hoher Sensitivität nachzuweisen. Die Methode empfiehlt sich insbesondere für die Analyse der sehr langsam ablaufenden anaeroben Abbauprozesse persistenter Verbindungen wie z.B. MCB und PAKs, da es kaum alternative Nachweisverfahren gibt. Damit steht neben der komponentenspezifischen Isotopenanalyse (CSIA) und *in situ* Tracerversuchen eine weitere innovative Methode für die Erfassung der *in situ* Biodegradation bei der Implementierung von *Natural Attenuation* als Standortsicherungskonzept in der Altlastenpraxis zur Verfügung. Der Einsatz des Testsystems ist in konventionell ausgebauten und Multilevel Messstellen sowie direkt im Sedimentkörper von zum Beispiel Pflanzenkläranlagen möglich. Die Methode der *in situ* Mikrokosmen wurde bereits im KORA-Leitfaden sowie der Methodensammlung über mikrobiologische NA-Untersuchungsverfahren (KORA, 2007; KORA, 2008) integriert und im Einverständnis mit den verantwortlichen Umweltbehörden an verschiedenen Standorten für den geforderten Nachweis der *in situ* Biodegradation im Rahmen von MNA Vorhaben angewendet.

Abstract

The natural ability to remove contaminants by microbial degradation is increasingly used as remedial alternative especially at heavily contaminated field sites (megasites*)* where active remediation technologies are often technically and economically not feasible. The implementation of Monitored Natural Attenuation (MNA) requires a careful evaluation of the ongoing biogeochemical processes under *in situ* conditions. The Natural Attenuation processes including various chemical-, physical- and biological processes may result in the reduction of the mass, toxicity, mobility, volume, or concentration of contaminants in the aquifer system. Beside the abiotic processes such as sorption, volatilization or dilution, *in situ* biodegradation is the only process leading to a sustainable removal of contaminants and therefore, microbial degradation is the most important sink of contaminants at contaminated field sites. Consequently, the implementation of Natural Attenuation as remediation strategy requires a careful site characterization with special attention regarding the *in situ* biodegradation processes.

Today, many different methods to determine the *in situ* biodegradation are available. Most of these conventional techniques only allow to indirectly assess the microbial degradation potential. Since the development of *in situ* microcosms (BACTRAP®), one further method is now available to directly provide evidence of *in situ* degradation beside compound-specific stable isotope analysis (CSIA) and *in situ* tracer tests.

Main objectives of this thesis were (I) to validate the new *in situ* microcosm test system for the assessment of *in situ* biodegradation, (II) the application of *in situ* microcosms at different field sites with different contaminants to evaluate the method with alternative techniques, (III) the investigation of the intrinsic degradation potential of persistent contaminants, (IV) the further development of the method to enable the analyses of the microbial consortia involved in contaminant degradation and (V) the reconstruction of degradation pathways by means of metabolite analyses. Additionally, (VI) the acceptance and implementation of the test system to determine *in situ* biodegradation within the context of Natural Attenuation as management strategy for contaminated field sites was addressed.

The application of *in situ* microcosms amended with ^{13}C-labelled substrates enabled the direct detection of *in situ* biodegradation of persistent compounds inside the contaminated aquifer within only a few months. During this thesis, the test system was successfully applied at different field sites to determine the anaerobic degradation of benzene, toluene, monochlorobenzene (MCB) as wells as of different polycyclic aromatic hydrocarbons (PAH). Many field studies were performed in close collaborations with industrial companies.

To characterise the anaerobic benzene degradation and to validate the *in situ* microcosm test system the vertical structure of a benzene plume at a former hydrogenation plant was analysed by means of geochemical and isotope chemical methods (chapter 2). All results were in good

agreement. At the highly contaminated zone of the plume the results of the CSIA did not show benzene degradation. Only the use of ^{13}C-labelled tracers in *in situ* microcosms enabled a very sensitive detection of the anaerobic degradation processes in all parts of the plume.

Using DNA fingerprinting methods it was shown that the microbial community colonizing the *in situ* microcosms mainly represents the groundwater microflora (chapter 3, 4). Further field studies to characterise the benzene and toluene degrading microorganisms revealed that the test system in combination with nucleic acid based molecular methods is appropriate to sensitively reflect the microbial community in accordance with the local geochemistry. The analysis of fatty acids alone was not sufficient for taxonomic interpretation.

Another indicator of *in situ* biodegradation is the proof of characteristic ^{13}C-labelled metabolites, which can also be used to elucidate degradation mechanisms. In this context, benzylsuccinate, a well known metabolite of the anaerobic toluene degradation (chapter 3) and benzene as intermediate of the reductive dehalogenation of MCB (chapter 7) were extracted and identified from *in situ* microcosms. The use of activated charcoal-like material allows the enrichment of metabolites on the test system. Analysing metabolite spectra will facilitate the reconstruction of metabolic pathways.

Using stable isotope techniques, for the first time complete mineralisation of MCB under anoxic conditions was proven (chapter 5, 6). Furthermore, anaerobic microbial degradation of MCB under *in situ* conditions was shown at different field sites applying the *in situ* microcosm test system (chapter 5-7). During these experiments MCB was considered as model compound representing persistent contaminants. The investigations at the field site in Bitterfeld were performed using a multilevel sampling approach (chapter 5) and inside a constructed wetland system, where for the first time *in situ* microcosms were directly incubated inside the sediment (chapter 7). Accordingly, it can be concluded that the test system is suitable for direct applications inside soils or sediments.

The direct proof of microbial degradation of single contaminants inside a multi component mixture is feasible using stable isotope techniques such as CSIA or *in situ* microcosms, even at highly complex field sites. To characterise the Natural Attenuation potential at a chlorobenzene contaminated aquifer an integrated approach was developed (chapter 6). Because the application of the Rayleigh concept to estimate the *in situ* biodegradation on the basis of the change in the isotope composition is limited for sequential degradation pathways, an isotope balance was determined. Beside the innovative isotope techniques multivariate statistics were used to analyse the data for the interpretation of the heterogeneous hydrogeochemical aquifer conditions. Despite the highly complex site conditions the integrative approach enabled to provide multiple lines of evidence for the ongoing *in situ* degradation processes as required by the authorities if Natural Attenuation will be considered as remediation option.

To summarize, in this thesis it is shown that the *in situ* microcosm test system, based on the use of ^{13}C-labelled tracer compounds, can be applied to sensitively determine the activity, structure and function of the microbial community involved in contaminant degradation within a reasonable time frame. The method is in particular recommended for the analysis of the very slow degradation processes of persistent compounds such as MCB and PAHs, since almost no alternative methods are available. Beside the CSIA and *in situ* tracer studies, now a further innovative method exists to prove *in situ* biodegradation according existing national and international guidelines (Hessisches Landesamt für Umwelt und Geologie, 2004; ITVA, 2004; KORA, 2007; KORA, 2008; US-EPA, 1999) for the implementation of Natural Attenuation as monitoring strategy for contaminated field sites. The test system can be used in conventional wells and in combination with multilevel sampling devices as well as directly inside the sediment as shown in investigations inside a constructed wetland. The concept of *in situ* microcosms to assess *in situ* biodegradation is already implemented in the “KORA guideline for Natural Attenuation investigation methods” (KORA, 2007; KORA, 2008) and was applied in agreement with the responsible environmental agencies at several field sites to provide the required information concerning the *in situ* degradation potential within the framework of Natural Attenuation.

1 Einleitung

1.1 Altlastenproblematik

Wasser gilt als Lebenselixier und die Verfügbarkeit von Wasser bildet einen entscheidenden Faktor für die Verbesserung der Lebensqualität sowie der ständig voranschreitenden wirtschaftlichen Entwicklung der Menschheit (WHO, 2006). Daher stellt die Versorgung der Weltbevölkerung mit hygienisch und toxikologisch unbedenklichem Trink- und Nutzwasser eine der größten Herausforderungen für die Zukunft dar. Die Nutzung von Grundwasservorkommen ist in vielen Gebieten der Erde wesentlicher Bestandteil der Wasserversorgung. In Deutschland stellt das Grundwasser sogar die Hauptquelle für die Trinkwassergewinnung dar. Jährlich werden rund 4 km^3 aus den Grundwasserleitern gefördert (BGR, Stand 2004). Seit der Industrialisierung im 19. Jahrhundert wurden zunehmend umweltrelevante Stoffe in den Untergrund eingetragen. Ein ausgeprägtes Umweltbewusstsein existierte zu dieser Zeit in der Öffentlichkeit noch nicht. Vor allem in Bereichen von Bergbaufolgelandschaften, ehemaligen Standorten der chemische Industrie und Metallurgie, landwirtschaftlich intensiv genutzte Flächen sowie in Gebieten mit militärischer Nutzung gelangten eine Vielzahl von Umweltschadstoffen durch unsachgemäßen Umgang, Leckagen, Havarien oder Kriegsschäden in den Boden und schließlich in das Grundwasser. In Folge dessen finden wir heute im Bereich ehemaliger Industriestandorte eine Vielzahl von Grundasserschadensfällen. Allein in Deutschland sind laut Umweltbundesamt rund 272.240 Altlastenverdachtstflächen registriert. Dabei handelt es sich um Standorte, bei denen der Verdacht schädlicher Bodenveränderungen oder sonstiger Gefahren für den Einzelnen oder die Allgemeinheit besteht (Umweltbundesamt, 2007). Eine spezielle Problematik stellen dabei die Standorte dar, die durch sehr großräumige Kontaminationen und umfangreiches Schadstoffinventar, so genannte Megasites, charakterisiert sind (Schirmer et al., 2006). Die Altlastenproblematik ist nicht nur ein deutsches oder europäisches Problem, sondern von globaler Relevanz.

1.2 Relevante Schadstoffe

Die bedeutendsten Grundwasserkontaminanten stellen die organischen Schadstoffe dar. Wie Arneth et al. in ihrer Studie gezeigt haben, gehören die chlorierten Kohlenwasserstoffe (CKW), im speziellen die aliphatischen, leichtflüchtigen halogenierten Kohlenwasserstoffe (LHKW) zu den am häufigsten vorkommenden Verunreinigungen in Deutschland (Arneth et al., 1989). Daneben sind vielfach auch Aromaten, wie z.B. Benzol, Toluol, Ethylbenzol und Xylol (BTEX) aber auch Chlorbenzole (CB) im Grundwasserabstrom von ehemaligen Produktionsstätten wie Hydrierwerken, Chemiefabriken, chemischen Reinigungen,

Imprägnierwerken u.ä. zu finden (Fig. 1-1). Des Weiteren sind polyzyklische aromatische Kohlenwasserstoffe (PAK) (Kanaly and Harayama, 2000) sowie Methyl-*tert*-butylether (MTBE) (Schmidt et al., 2002) relevante Grundwasserschadstoffe. Eine generell ähnliche Situation finden wir auch in den USA, wie die Studie von Plumb & Pitchfork (Plumb JR and Pitchford, 1985) zeigt und wie in der vergleichenden Gegenüberstellung der Daten von Deutschland und den USA zu entnehmen ist (Arneth et al., 1989; Teutsch et al., 1997).

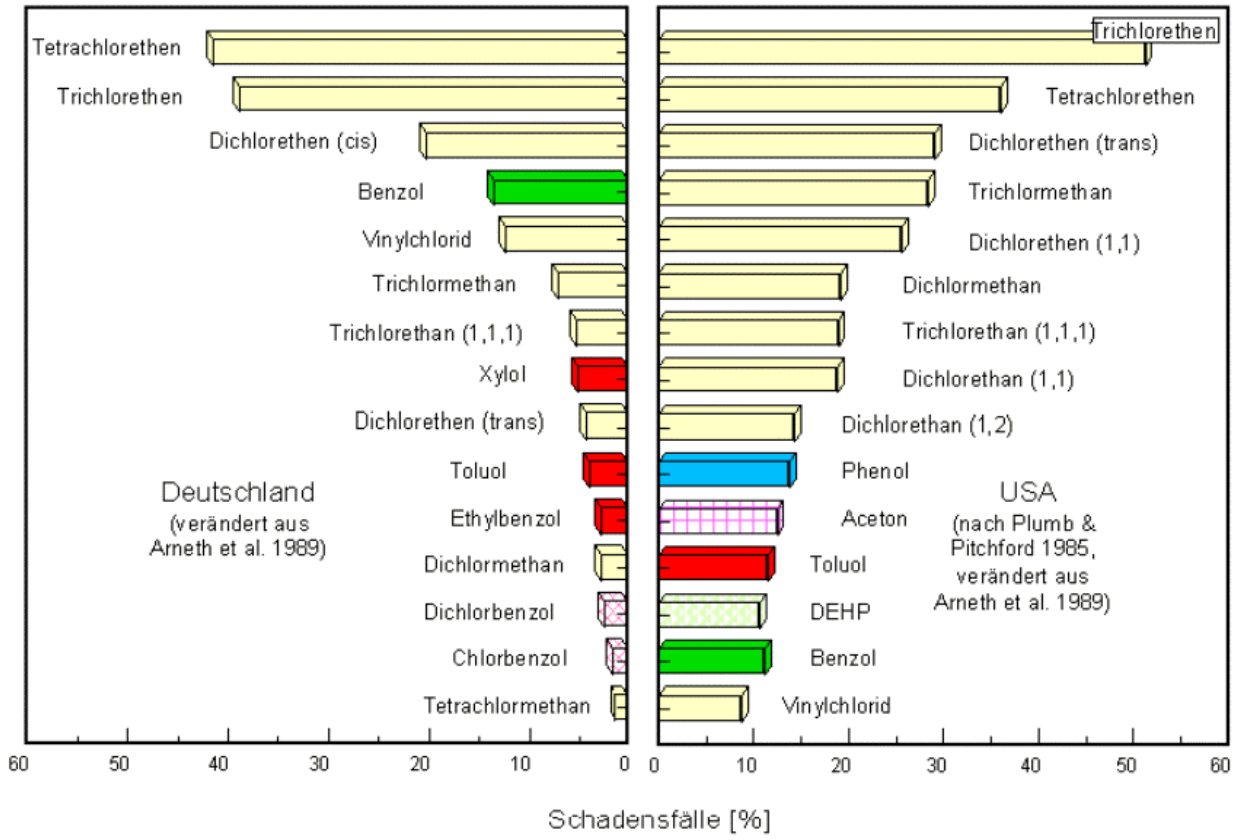

Fig. 1-1: Ländervergleich Deutschland und USA über das Vorkommen und die Häufigkeit organischer Schadstoffe im Abstrom von Schadensfällen aus (Teutsch et al., 1997).

Ein Großteil dieser xenobiotischen Verbindungen ist durch hohe Toxizität, Persistenz sowie karzinogene Eigenschaften gekennzeichnet, womit sich ihre hohe Umweltrelevanz begründet. Neben den organischen Schadstoffen gibt es auch Verunreinigungen mit anorganischen Verbindungen (Schwermetalle, Nitrat, etc.). Den Schwerpunkt der vorliegenden Arbeit bilden jedoch die organischen Kontaminanten. Grundwasserschadensfälle mit anorganischen Verbindungen werden nicht näher behandelt.
Die physiko-chemischen Eigenschaften und die Geologie eines Standortes sowie die damit im Zusammenhang stehenden Prozesse Advektion, Dispersion, Diffusion, Sorption, Verflüchtigung und Abbau kontrollieren das Ausbreitungsverhalten der Schadstoffe im Grundwasser. In Abhängigkeit von der Wasserlöslichkeit, dem Sorptionsverhalten und der biologischen Abbaubarkeit unterscheidet sich das Transportverhalten der verschiedenen Schadstoffe. Kontaminanten mit sehr guter Wasserlöslichkeit, geringer Retardation und

hoher Persistenz sind oft durch große Transportweiten gekennzeichnet, so dass sich die Schadstoffe über weite Strecken im Untergrund entlang der Grundwasserfließrichtung ausbreiten. Zu dieser Stoffgruppe zählen beispielsweise die CKW, die Fahnenlängen von mehreren Kilometern aufweisen können. Die Transportweite der BTEX hingegen ist, trotz relativ guter Wasserlöslichkeit und geringer Sorption, infolge guter Abbaubarkeit mit Fahnenlängen von meist weniger als 500 m wesentlich geringer. Die PAK hingegen zeichnen sich durch eine weitaus geringere Wasserlöslichkeit und damit verbundene stärkere Sorption an der Aquifermatrix aus, die in Abhängigkeit der Anzahl der Benzolringe variiert. Fahnenlängen von PAK Schadensfällen sind daher abhängig vom PAK-Inventar und können um mehrere Größenordnungen variieren (Teutsch et al., 1997).
Da von kontaminiertem Grundwasser ein hohes Gefährdungspotenzial für den Mensch und die Umwelt ausgehen kann, ist die Überwachung und gegebenenfalls Sanierung von großem öffentlichem Interesse. Die *in situ* Biodegradation kann wesentlich zur nachhaltigen Entfernung der Schadstoffmengen im kontaminierten Grundwasser beitragen. Deshalb ist die Charakterisierung der biogeochemischen Prozesse im Aquifer für ein differenziertes Prozessverständnis ein erster wichtiger Schritt, bevor Entscheidungen bezüglich geeigneter Sanierungsmaßnahmen bzw. Standortmanagementstrategien getroffen werden können.

1.3 Natürliche Selbstreinigung: *Natural Attenuation*

Die Berücksichtigung der natürlichen Rückhalte- und Abbauprozesse gewinnt in der Altlastenbearbeitung zunehmend an Bedeutung (Grandel and Dahmke, 2004; Rittmann, 2004). Die rechtliche Grundlage hierfür bildet in Deutschland das am 1. März 1999 in Kraft getretene Bundes-Bodenschutzgesetz (BBodSchG). Insbesondere im Fall von Schadensfällen, bei denen die Kosten und der Nutzen einer Sanierung im herkömmlichen Sinn (z.B. *Pump & Treat*, Reaktive Wände sowie *Funnel & Gate*) in keinem günstigen Verhältnis stehen und zu keiner nachhaltigen Dekontamination des Standortes führen, werden immer häufiger die natürlichen Selbstreinigungskräfte des Aquifers in Betracht gezogen, um eine langfristige Sicherung des Standortes zu erreichen. An eine zunächst aktive Sanierung (z.B. Quell- oder Hot-Spot Sanierung) kann sich eine passive Überwachung und Sicherung des Standortes durch *Monitored Natural Attenuation* (MNA) anschließen.
Natürliche Abbau- und Rückhalteprozesse werden unter den Begriff *Natural Attenuation* (NA) zusammengefasst und verlangsamen die Schadstoffausbreitung im Grundwasserleiter, weshalb Schadstofffahnen nur eine begrenzte Ausdehnung im Aquifer erreichen (Wiedemeier et al., 1999). Die amerikanische Umweltbehörde (US-EPA) definiert *Natural Attenuation als Eigenschaft eines Aquifers durch Prozesse wie biologischer Abbau, Dispersion, Verdünnung, Sorption, Verflüchtigung und/oder chemischer oder biochemischer Stabilisierung die Masse, Toxizität, Mobilität oder das Volumen der Schadstoffe so weit zu*

reduzieren, dass die menschliche Gesundheit und das Ökosystem nicht gefährdet sind (US-EPA, 1999).

Den Schwerpunkt von NA als Sicherungsmaßnahme bilden gezielte Untersuchungen zum Nachweis der natürlichen Abbau- und Rückhalteprozesse am jeweiligen Standort in Verbindung mit einem sich anschließenden Langzeitmonitoring (MNA). Der mikrobielle Schadstoffabbau (*intrinsic biodegradation*) ist ein wesentlicher Prozess im Zusammenhang mit NA, da sich infolge der Biodegradation nicht nur die Konzentrationen sondern auch die Schadstofffrachten im Grundwasser reduzieren. Die gezielte Stimulation des Abbaupotenzials (*Enhanced Natural Attenuation - ENA*) durch Zugabe von z.B. Elektronenakzeptoren kann die Effektivität der NA-Prozesse steigern, wie zum Beispiel an verschiedenen Tetrachlorethen (PCE) kontaminierten Standorten gezeigt werden konnte (Major et al., 2002; Ramsburg et al., 2004). Eine umfangreiche Zusammenfassung zu aktuellen Entwicklungen bezüglich NA und ENA Maßnahmen wurde anhand verschiedener Standorte mit chlorierten Ethenen, aromatischen Kohlenwasserstoffen bzw. MTBE als Hauptkontaminanten in Übersichtsartikeln veröffentlicht (Farhadiana et al., 2008; Jørgensena, 2007; Scow and Hicks, 2005).

In den USA hat sich MNA als Alternative zu aktiven Sanierungsmaßnahmen speziell für Schadensfälle mit MKW bereits etabliert, was sich auch in den bestehenden Handlungsempfehlungen der amerikanischen Umweltbehörde US-EPA zur Anwendung von NA widerspiegelt (Doll, 1998; US-EPA, 1999; US-EPA, 2004; Wöstmann, 2007). In Deutschland hingegen fehlten in der Vergangenheit die rechtlichen und fachlichen Grundlagen für eine sachgerechte Implementierung und gezielte Berücksichtigung der natürlichen Selbstreinigungskräfte in der Altlastensanierung (Wöstmann, 2007). NA war und ist daher Inhalt verschiedener deutscher Forschungsprojekte, wie zum Beispiel der vom BMBF geförderte Förderschwerpunkt „KORA - Kontrollierter natürlicher Rückhalt und Abbau von Schadstoffen bei der Sanierung kontaminierter Böden und Grundwässer“ oder auch verschiedene Langzeituntersuchungen des Umweltbundesamtes (BMBF, 2000). Als Ergebnis dieser umfangreichen Forschungstätigkeiten liegen heute verschiedene Merkblätter, Handlungsempfehlungen und Arbeitshilfen vor, die den verantwortlichen Behörden, Ingenieurbüros und Sanierungspflichtigen als Leitfäden und Arbeitsrichtlinien zur Verfügung stehen. In diesem Kontext sind die folgenden Schriften zu nennen, wobei diese Liste keinen Anspruch auf Vollständigkeit erhebt (Bayrisches Landesamt für Wasserwirtschaft, 2004; Hessisches Landesamt für Umwelt und Geologie, 2004; ITVA, 2004; KORA, 2007; LABO, 2005):

- Bayrisches Landesamt für Wasserwirtschaft (2004): Natürliche Schadstoffminderung bei Grundwasserverunreinigungen durch Altlasten und schädliche

Bodenveränderungen - Natural Attenuation - Merkblatt Nr. 3.8/3 vom 05.11.2004, München, 17 S.

- Hessisches Landesamt für Umwelt und Geologie (2004): Arbeitshilfe zu überwachten natürlichen Abbau- und Rückhalteprozessen im Grundwasser (Monitored Natural Attenuation MNA), Wiesbaden, Hessisches Landesamt für Umwelt und Geologie. Handbuch Altlasten, Bd.8, T.1
- ITVA (2004): Monitored Natural Attenuation. Ingenieurtechnischer Verband Altlasten e.V., ITVA-Arbeitshilfe –H1-12, Dezember 2004, Berlin, 35 S.
- LABO (2005): Berücksichtigung natürlicher Schadstoffminderungsprozesse bei der Altlastenbearbeitung -Bund-/Länderarbeitsgemeinschaft Bodenschutz (LABO) – Altlastenausschuss (ALA) Ad-hoc Unteraussschuss „Natural Attenuation“, vom 01.06.2005
- KORA (2007): Mikrobiologische NA-Untersuchungsmethoden: Fachliche Grundlagen für die Anwendung von Methoden zur Erfassung des natürlichen mikrobiellen Schadstoffabbaus im Aquifer / ProcessNet, Expertengruppe "Intrinsisches Abbaupotenzial" im Arbeitsausschuss "Ressourcenmanagement Boden und Grundwasser". - Stand: März 2007. – Frankfurt M. DECHEMA, 2007. - 73 S.

Diese Schriftreihen dienen als Richtlinien zur Berücksichtigung natürlicher Abbau- und Rückhalteprozesse in kontaminierten Aquiferen in der Sanierungspraxis und sie unterstreichen die Bedeutung der mikrobiell initiierten Prozesse, die einen signifikanten Beitrag zur nachhaltigen Reduzierung der Schadstoffmengen leisten können.

1.4 Nachweis der *in situ* Biodegradation

Der Nachweis des biologischen Abbaus organischer Verbindungen ist ein wesentliches Element für die Implementierung von Natural Attenuation zur Überwachung und Sicherung kontaminierter Standorte, da nur der mikrobielle Schadstoffabbau zu einer destruktiven und vor allem nachhaltigen Reduzierung der Schadstoffmengen im Grundwasser beiträgt. Abiotische Prozesse, wie zum Beispiel die Sorption und Verflüchtigung sind für eine Gefahrenabwehr nicht unbedingt akzeptabel, da es hierbei nur zu einer Verlagerung der Kontaminanten in andere Umweltkompartimente kommt und keine tatsächliche Reduzierung der Schadstofffrachten erfolgt. Daher ist es von besonderem Interesse, geeignete Methoden zur Charakterisierung des *in situ* Abbaupotenzials kontaminierter Aquifere zu entwickeln. Einen Überblick zu traditionellen und innovativen NA-Untersuchungsverfahren gibt die Broschüre, die im Rahmen des BMBF geförderten KORA-Forschungsvorhaben entstanden ist (KORA, 2007).

Der Nachweis der *in situ* Biodegradation kann anhand hydrogeochemischer Indikatoren erfolgen. Dabei wird der Rückgang der Schadstoffkonzentrationen mit der Ausbildung spezifischer Redoxzonen relevanter terminaler Elektronenakzeptoren (TEA) in Beziehung gebracht. Ein gleichzeitiges Auftreten von charakteristischen Metaboliten untermauert hierbei die Existenz des *in situ* Abbaupotenzials. Des Weiteren gehören zu den mikrobiologischen Indikatoren für *in situ* Abbau (1) die Bestimmung von stoffwechselspezifischen Keimzahlen mit der Most-Probable-Number-(MPN)-Methode, (2) mikrobiologische Untersuchungen an Standortmaterial im Rahmen von Labormikrokosmenstudien, sowie (3) der Nachweis über das Vorhandensein von abbauaktiven Mikroorganismen mithilfe von „Fingerprinting“ Verfahren. Bei diesen Techniken handelt es sich um indirekte Nachweisverfahren. Der Nachweis erfolgt meist *ex situ*, also in Laborstudien, so dass eine direkte Überführung der Ergebnisse auf die realen komplexen Aquiferbedingungen schwierig bleibt. Auch die Identifizierung von Mikroorganismen von denen bekannt ist, dass sie zum Abbau von bestimmten Kontaminanten befähigt sind, ist noch kein eindeutiger Beweis, dass diese Bakterien auch tatsächlich am jeweiligen Untersuchungsstandort am Abbau beteiligt sind. Insbesondere da die Mehrheit der Mikroorganismen noch nicht kultiviert wurde (Amann et al., 1995) und auch die Abbauwege bestimmter Schadstoffe (z.B. Benzol, Monochlorbenzol) bis heute noch nicht verstanden sind, ist ein zweifelsfreier Nachweis der *in situ* Abbauaktiviät mit diesen konventionellen Methoden schwierig bzw. kaum möglich. Deutlich bessere Aussagekraft und einen sehr hohen Informationsgehalt bieten die Isotopenmethoden.

Bei der Analyse der substratspezifischen Isotopenfraktionierung werden anhand der Veränderung der Isotopensignaturen eines Schadstoffes Rückschlüsse zum Abbaupotenzial gezogen und der *in situ* Abbau kann über einen Fließweg erfasst werden. Da Mikroorganismen aus energetischen Gründen bevorzugt isotopisch leichtes Substrat verwerten, kommt es in der noch nicht abgebauten residualen Schadstofffraktion zu einer relativen Anreicherung der schweren Isotope. Anhand dieser Verschiebung der natürlichen Isotopenverhältnisse kann unter Kenntnis der spezifischen Isotopenfraktionierungsfaktoren die *in situ* Biodegradation auch quantitativ erfasst werden (Meckenstock et al., 2004a; Meckenstock et al., 1999) (siehe auch Kapitel 1.4.1).

Die Charakterisierung des mikrobiellen Abbaus im Aquifer kann auch anhand von *in situ* Tracerexperimenten in einer Grundwassermessstelle (Push- und Pull Versuche) (Pombo et al., 2002) oder entlang einer Fließstrecke erfolgen. Dabei wird ein isotopisch markierter Schadstoff (z.B. Deuterium markiertes Toluol) sowie ein konservativer Tracer in das Grundwasser eingespeist. Mithilfe unterschiedlicher Konzepte wie Isotopenfraktionierung, Analyse von Mineralisierungsprodukten und Metaboliten können anschließend quantitative Aussagen hinsichtlich des Abbaus getroffen werden (Fischer et al., 2006; Reusser and Field, 2002) (siehe auch Kapitel 1.4.2).

Da der biologische Abbau die wichtigste und nachhaltigste Schadstoffsenke darstellt, ist der Nachweise der mikrobiellen Abbauprozesse von großer Bedeutung für die Charakterisierung und Bewertung des NA Potenzials eines Standortes. Wie in diesem Kapitel beschrieben wurde, existieren inzwischen eine Vielzahl verschiedener Methoden, um den Nachweis der Biodegradation zu führen und umfangreiche Aussagen hinsichtlich der Schadstoff mindernden Prozesse zu erhalten. Ein wesentlicher Nachteil, der sich mit dem Großteil der bisher beschriebenen Methoden verknüpft, ist jedoch die Tatsache, dass oftmals zwar das Abbaupotenzial analysiert wird, Indikatoren über die tatsächliche Abbauaktivität jedoch nicht geliefert werden können, denn allein das Vorhandensein von zum Abbau befähigten Mikroorganismen sagt nichts über deren tatsächliche Abbauaktivität am entsprechenden Standort aus. Mit Ausnahme der Isotopenmethoden (Isotopenfraktionierung, Tracertests) handelt es sich bei allen anderen Konzepten um so genannte indirekte Nachweisverfahren, die keine spezifischen Informationen über die *in situ* Aktivität der Mikroorganismen liefern. Nur die auf Isotopen basierenden Methoden ermöglichen den direkten Nachweis des mikrobiellen Schadstoffabbaus. Da die Durchführung von *in situ* Tracerversuchen mit enormem Kosten- und Arbeitsaufwand verbunden ist und darüber hinaus eine Genehmigung zur Einleitung von Schadstoffen benötigt wird, ist dieses Verfahren sicher nur in einzelnen Fällen für die Altlastenpraxis geeignet. Auch wenn die Methode der Isotopenfraktionierung seit den letzten Jahren stets an Bedeutung gewonnen hat, sind alternative Methoden notwendig, um den geforderten Nachweis über die *in situ* Abbauaktivität an kontaminierten Standorten zu erbringen.

In diesem Zusammenhang wurde ein weiteres direktes Testverfahren (*in situ* Mikrokosmen – BACTRAP®) entwickelt, welches auf dem Einsatz stabiler Isotope als Tracer basiert (Kapitel 1.5). In den folgenden Kapiteln soll zunächst etwas detaillierter in die Grundlagen ausgewählter Methoden (Isotopenfraktionierung, Tracerversuche, Molekularbiologische Methoden, *in situ* Mikrokosmen) eingeführt werden, bevor anschließend in den einzelnen Kapiteln dieser Promotionsarbeit der Stand der Forschung zur Anwendung von *in situ* Mikrokosmen zum Nachweis des mikrobiellen Schadstoffabbaus präsentiert und diskutiert wird (Kapitel 2 bis 7).

1.4.1 Stabile Isotopenfraktionierung

Eine Möglichkeit zur Untersuchung des *in situ* Abbaus von Schadstoffen bietet die Methode der Isotopenfraktionierung, bekannt als „stabile Isotopenfraktionierungsanalyse“ (SIFA) oder „substratspezifische Isotopenfraktionierungsanalyse“ (*compound-specific stable isotope analysis*, CSIA). Umfangreiche Übersichtsartikel bieten eine fundierte Einleitung in die Thematik, insbesondere zu den Grundprinzipien der stabilen Isotopenchemie, der Analytik sowie zur Quantifizierung des mikrobiellen Abbaus organischer Schadstoffe am Beispiel von

Labor- und Feldstudien (Blessing et al., 2008; Meckenstock et al., 2004a; Schmidt et al., 2004).

Die überwiegende Mehrheit der natürlich vorkommenden Elemente besitzt mehrere stabile Isotope, die sich bei gleicher Ordnungszahl hinsichtlich ihrer Massenzahlen unterscheiden. Diese Massendifferenz beruht auf der unterschiedlichen Anzahl von Neutronen im Atomkern. Für Fragestellungen bezüglich des Nachweises der *in situ* Biodegradation von organischen Schadstoffen sind insbesondere Wasserstoff- (^{1}H, ^{2}H) und Kohlenstoffisotope (^{12}C, ^{13}C) von Bedeutung, da organische Kontaminanten im Wesentlichen aus diesen beiden Elementen aufgebaut sind. Die verschiedenen Isotope eines Elementes unterscheiden sich signifikant hinsichtlich ihrer relativen Häufigkeit mit der sie in der Umwelt vorkommen (Tab. 1-1).

Tab. 1-1: Relative Häufigkeiten [%] der wichtigsten stabilen Isotope ausgewählter Elemente und Angabe der internationalen Standards (Hoefs, 1997)

Element	Isotope	Relative Häufigkeit [%]	Internationaler Standard
Wasserstoff	$^{1}H/^{2}H$ (H/D)	99,98 / 0,02	V-SMOW
Kohlenstoff	$^{12}C/^{13}C$	99,89 / 1,11	V-PDB
Stickstoff	$^{14}N/^{15}N$	99,64 / 0,36	Luft
Sauerstoff	$^{16}O/^{18}O$	99,76 / 0,02	V-SMOW
Schwefel	$^{32}S/^{34}S$	94,02 / 4,21	V-CTD
Chlor	$^{35}Cl/^{37}Cl$	75,53 / 24,47	V-SMOC

Die Methode der Isotopenfraktionierung beruht darauf, dass der mikrobielle Schadstoffabbau mit einem kinetischen Isotopenfraktionierungseffekt verbunden ist, weshalb das natürliche Isotopenverhältnis (z.B. $^{13}C/^{12}C$) eines Substrates im Verlauf der Abbaureaktion verändert wird. Die Ursache der Isotopenfraktionierung liegt in den unterschiedlichen Aktivierungsenergien (Unterschied der Nullpunktschwingungen), die benötigt werden, um chemische Bindungen zu spalten oder aufzubauen: die Spaltung (oder Bildung) einer Bindung mit schweren Isotopen erfordert höhere Aktivierungsenergien, so dass bevorzugt leichte Isotopomere einer Kontaminante während der mikrobiellen Umsetzung verwertet werden, wodurch es zu einer Anreicherung der schweren Isotopomere in der residualen, nicht abgebauten Schadstofffraktion kommt. Die daraus resultierenden Änderungen der Isotopenverhältnisse werden mit Hilfe der Gaschromatographie-Isotopenverhältnis-Massenspektroskopie (GC-IRMS) analytisch nachgewiesen. Derzeit sind online Messungen mit GC-IRMS für Kohlenstoff, Wasserstoff, Stickstoff und Sauerstoff möglich (Brenna et al., 1997). Die Reproduzierbarkeit der Messungen liegt für Kohlenstoffisotopenanalysen bei 0,2 bis 0,5 ‰ und für Wasserstoffisotopenanalysen bei 2 bis 5 ‰ (Meckenstock et al., 2004a). Die Isotopensignatur einer Verbindung wird in Promille [‰] relativ zu einem internationalen Standard in der so genannten Delta-Notation angegeben (Gleichung 1-1) (Coplen et al., 2006).

Gleichung (1-1)
$$\delta^{13}C\,[‰] = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample} - \left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}}{\left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}} \times 1000$$

Die Rayleigh-Gleichung (Gleichung 1-2) beschreibt den mathematischen Zusammenhang zwischen der Konzentrationsänderung der mikrobiell umgesetzten Substanz in Verbindung mit der Änderung der Isotopensignatur durch den Isotopenfraktionierungsfaktor (α) (Hoefs, 1997).

Gleichung (1-2)
$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\left(\frac{1}{\alpha}-1\right)}$$

R_0 und R_t geben die Isotopenzusammensetzung, C_0 und C_t die Konzentration des Substrats zum Zeitpunkt 0 und t der mikrobiellen Umsetzung an. Reaktionsspezifische Isotopenfraktionierungsfaktoren werden unter definierten Laborbedingungen bestimmt, um zu garantieren, dass ausschließlich die Biotransformationsreaktion die Veränderung von Konzentration und Isotopensignatur des Substrats verursachen. Eine Übersicht bereits publizierter Fraktionierungsfaktoren ist in der Datenbank der Firma Isodetect im Internet hinterlegt, die regelmäßig aktualisiert wird (www.isodetect.de).
Anhand der Verschiebung der natürlichen Isotopenverhältnisse kann unter Kenntnis der spezifischen Isotopenfraktionierungsfaktoren (α) die *in situ* Biodegradation auch quantitativ erfasst werden (Meckenstock et al., 2004a; Meckenstock et al., 1999). Der prozentuale biologische Abbau B[%] wird dabei über den Zusammenhang der Rayleigh Gleichung berechnet (Gleichung 1-3) (Meckenstock et al., 2002).

Gleichung (1-3):
$$B[\%] = \left(1 - \frac{C_t}{C_0}\right) * 100 = \left(1 - \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\frac{1}{\alpha}-1}\right)}\right) * 100$$

CSIA wurde bereits erfolgreich an einer Vielzahl von Feldstandorten für BTEX (z.B. Griebler et al., 2004b; Mancini et al., 2002; Vieth et al., 2005), chlorierte Ethene (z.B. Hunkeler et al., 1999a; Imfeld et al., 2008; Nijenhuis et al., 2005; Sherwood Lollar and Slater, 2001) und MTBE (z.B. Kuder et al., 2005; Rosell et al., 2007b; Zwank et al., 2005) zur Charakterisierung des *in situ* Abbaupotenzials angewendet. Für abiotische Prozesse wie z.B. Sorption oder Verdünnung konnte gezeigt werden, dass sie unter den *in situ* Bedingungen nicht mit einem signifikanten Isotopenfraktionierungseffekt assoziiert sind (Kopinke et al.,

2005; Meckenstock et al., 1999; Slater et al., 2000). Des Weiteren ermöglicht die mehrdimensionale Isotopenfraktionierungsanalyse (gleichzeitige Betrachtung von z.B. Kohlenstoff- und Wasserstoffisotopen) Rückschlüsse über die Abbauwege zu ziehen (Elsner et al., 2007; Fischer et al., 2008; Fischer et al., 2007).

Das Ausmaß der Isotopenfraktionierung wird hauptsächlich durch den Reaktionsmechanismus des initialen Angriffs auf das Substrat bestimmt (primärer Isotopeneffekt), wie beispielsweise Meckenstock et al. (1999) für den Toluolabbau gezeigt haben. Sekundäre Isotopeneffekte sind in der Regel ein bis zwei Größenordnungen geringer und beschreiben die Fraktionierung von Isotopen, die nicht direkt in der Abbaureaktion involviert sind.

Ein weiterer Einflussfaktor ist die Molekülgröße. Ein signifikanter Isotopeneffekt findet nur an der Position im Molekül statt, wo die biochemische Reaktion erfolgt. Bei der Analyse der Isotopenverhältnisse einer Verbindung werden allerdings alle Atome eines Moleküls erfasst, weshalb das Ausmaß der Isotopenfraktionierung in Abhängigkeit von der Molekülgröße abgeschwächt wird. Je größer das Molekül ist, umso kleiner ist der nachweisbare Isotopeneffekt. Für Schadstoffe mit großen Molekülen (> 12-14 Kohlenstoffatome), wie zum Beispiel höher molekulare PAKs, ist die Methode der Isotopenfraktionierung daher weniger geeignet, da der messbare Isotopeneffekt zu gering ist, um sensitiv genug nachgewiesen werden zu können (Meckenstock et al., 2004a).

In diesem Zusammenhang unterscheidet man zwischen dem intrinsischen Isotopeneffekt, der die tatsächliche Reaktion im Molekül widerspiegelt und dem kumulativen Isotopeneffekt für das gesamte Molekül, der in der Regel geringer ist (Elsner et al., 2005; Meckenstock et al., 2004a).

Aktuelle Studien haben gezeigt, dass die Isotopenfraktionierung während des mikrobiellen Abbaus organischer Schadstoffe neben dem Reaktionsmechanismus auch von anderen Faktoren, wie zum Beispiel der Temperatur (Morasch et al., 2001), den Enzymen (Morasch et al., 2004; Nikolausz et al., 2006), den am Abbau beteiligten Mikroorganismen sowie ratenlimitierenden Prozessen (z.B. Substrataufnahme und -transport in die Zelle) (Cichocka et al., 2008; Nijenhuis et al., 2005) beeinflusst werden können. Dies führt zu Limitationen für die Anwendung von CSIA zur Erfassung der *in situ* Biodegradation kontaminierter Standorte, da die Wahl eines geeigneten Fraktionierungsfaktors erschwert ist. Eine konservative Abschätzung der *in situ* Biodegradation ist dennoch möglich, insofern die Berechnungen mit den größten Fraktionierungsfaktoren durchgeführt werden, um eine Überschätzung des Abbaupotenzials zu vermeiden (Fischer et al., 2008; Rosell et al., 2007a).

1.4.2 Tracerversuche

Mithilfe von Mikrokosmenstudien im Labor kann der Abbau organischer Schadstoffe unter definierten Bedingungen nachgewiesen werden, in dem die Abnahme der

Schadstoffkonzentrationen und der gleichzeitige Verbrauch von Elektronenakzeptoren sowie die Bildung von Metaboliten als Indikatoren für Biodegradation dienen. Insbesondere für den Nachweis der Biodegradation einzelner Substanzen in einem Mix verschiedener Kontaminanten, die häufig im Bereich ehemaliger Altlasten zu finden sind, ist diese Methode jedoch ungeeignet, da verschiedene Elektronendonor und –akzeptorprozesse gleichzeitig ablaufen, was die Berechnung von Elektronenbilanzen erschwert. Besonderes Interesse besteht darin, die vollständige Mineralisierung eines Substrats beziehungsweise die Transformation in die Biomasse sowie Metabolite nachzuweisen. Hierfür eignen sich Tracerversuche mit isotopisch markierten Substraten (^{13}C, ^{14}C), bei denen anhand der Produktion von markiertem CO_2 die vollständige Mineralisierung eines bestimmten Schadstoffs gezeigt oder anhand der Inkorporation des markierten Kohlenstoffs in die Biomasse der biologische Abbau nachgewiesen werden kann.

Die Analyse von $^{14}CO_2$ ermöglicht einen sehr sensitiven Nachweis der vollständigen Mineralisierung (Bradley et al., 2002; Chapelle et al., 1996; Fent et al., 2003; Scheunert et al., 1987). Daher sind Tracerversuche besonders für Untersuchungen zum Verhalten und Verbleib von Pestiziden (Mordaunt et al., 2005) sowie persistenten umweltrelevanten Stoffen geeignet. Der Einsatz radioaktiver Tracer birgt jedoch Gefahren für Mensch und Umwelt und strenge Sicherheitsvorkehrungen erschweren die Arbeiten im Labor und im Feldeinsatz. Aufgrund der Umweltbedenken gibt es nur relativ wenige Feldanwendungen mit ^{14}C-markierten Tracerverbindungen (Bianchin et al., 2006; Rügge et al., 1999). Alternativ zu den radioaktiven Markierungen werden zunehmend stabile Isotope (z.B. ^{13}C-markierte Substrate, deuterierte Verbindungen) verwendet (Richnow et al., 1999a). Der wesentliche Vorteil stabiler Isotope liegt in der einfachen Handhabung sowie der Anwendbarkeit in offenen Systemen, so dass für *in situ* Untersuchungen, wie die Erfassung des mikrobiellen Schadstoffabbaus kontaminierter Aquifere, alternativ stabile Isotope als Tracer genutzt werden sollten.

In situ Tracerversuche eignen sich zur Charakterisierung des Transport- und Reaktionsverhaltens eines Grundwasserleiters (Thierrin et al., 1995). Zum Einsatz kommen sowohl konservative (z.B. Uranin, Bromid) als auch reaktive Tracer (der zu untersuchende Schadstoff isotopisch markiert). Prinzipiell können zwei Methoden unterschieden werden: (1) so genannte Push- und Pull Tests, bei denen die Tracer zunächst in einer Messstelle eingeleitet und nach einer gewissen Verweildauer aus der gleichen Messstelle extrahiert werden (Istok et al., 1997; Pombo et al., 2002; Schroth et al., 2001) oder (2) Tracerversuche, bei denen die Einspeisung der Tracer an einem Injektionsbrunnen erfolgt und die Ausbreitung entlang des Fließpfades in so genannten Kontrollbrunnen überwacht wird (Fischer et al., 2006). Mithilfe unterschiedlicher Konzepte wie Isotopenfraktionierung, Analyse von Mineralisierungsprodukten und Metaboliten können anschließend quantitative

Aussagen hinsichtlich des Abbaus getroffen werden (Fischer et al., 2006; Reusser and Field, 2002). Basierend auf dem Einsatz stabiler Isotope können anhand der Inkorporation markierten Kohlenstoffs in die Zellbestandteile der bakteriellen Biomasse Informationen bezüglich der am Schadstoffabbau beteiligten Mikroorganismen gewonnen (Dumont and Murell, 2005; Evershed et al., 2006; Friedrich, 2006b; Madsen, 2006; Radajewski et al., 2003) (siehe auch Kapitel 1.4.3) sowie anhand der Analyse von charakteristischen Metaboliten Abbauwege rekonstruiert werden (Reusser and Field, 2002).
Aufbauend auf dem Konzept stabile Isotope als Tracer zur Erfassung mikrobieller Aktivität zu nutzen, entwickelten Geyer et al. *in situ* Mikrokosmen, die einen direkten Nachweis der Biodegradation im kontaminierten Aquifer anhand der Inkorporation ^{13}C-markierten Kohlenstoffs in die Biomasse ermöglichen (Geyer et al., 2005).

1.4.3 Molekularbiologische Methoden

Mikroorganismen besitzen genetisch und phylogenetisch die größte Diversität unter den Lebewesen. Bisher sind jedoch nur etwa 5000 von weltweit schätzungsweise einer Milliarde existierenden Mikroorganismen kultivierbar (Amann et al., 1995). Das verdeutlicht, welche Schwierigkeiten mit den klassischen Methoden der Kultivierung verbunden sind (Fig. 1-2). Mithilfe von kultivierungsunabhängigen Methoden wie mikroskopischen Untersuchungen, Durchflusszytometrie *(flow cytometry)*, Fluoreszenz-*in-situ*-Hybridisierung (FISH), Polymerasekettenreaktion (PCR), DNA *Fingerprinting* Methoden (z.B. denaturierende Gradientengelelektrophorese, DGGE; DNA-Einzelstrang-Konformations-Polymorphismus-Analyse, *single strand conformation polymorphism analysis*, SSCP) sowie Stabile Isotopenbeprobung (SIP) ist es dennoch möglich Informationen über die Struktur und Bedeutung mikrobieller Gemeinschaften zu gewinnen (Fig. 1-2). Eine Vielzahl aktueller Übersichtsartikel geben eine gute Einführung zu den einzelnen Methoden und vergleichen die Vor- und Nachteile, die sich bei den unterschiedlichen Techniken je nach Fragestellung ergeben (Madsen, 2000; Spiegelman et al., 2005; Wagner et al., 2006; Weiss and Cozzarelli, 2008; Widada et al., 2002).
Molekularbiologische Methoden (Fig. 1-2) bieten die Möglichkeit, die Struktur und Diversität der mikrobiellen Gemeinschaft zu erfassen (Imfeld et al., 2008). Darüber hinaus können Gene spezifischer Enzyme auch als funktionelle Marker benutzt werden (Zhou, 2003), um Informationen bezüglich der Abbauwege bestimmter Schadstoffe zu gewinnen und das *in situ* Abbaupotenzial kontaminierter Systeme zu beurteilen (He et al., 2007; Kuntze et al., 2008; Winderl et al., 2007).

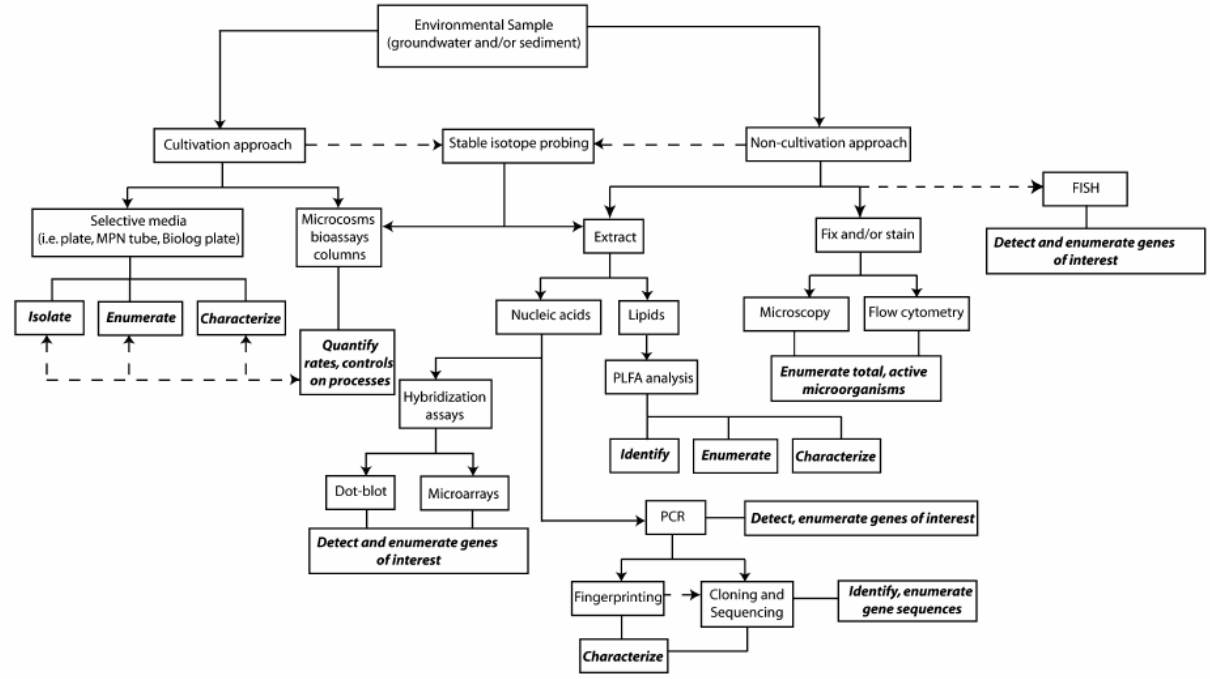

Fig. 1-2: Übersicht kultivierungsabhängiger und -unabhängiger Methoden zur Charakterisierung mikrobieller Gemeinschaften (Weiss and Cozzarelli, 2008).

Die vor allem in den letzten Jahren weiterentwickelte Methode der „Stabilen Isotopenbeprobung“ (SIP) von Nukleinsäuren ermöglicht darüber hinaus nicht nur die Diversität der mikrobiellen Gemeinschaften, sondern auch die metabolischen Fähigkeiten der bisher noch unkultivierten Mikroorganismen aufzuklären. Die Methode des SIP bietet daher den Schlüssel, um die Black Box mikrobieller Diversität zu öffnen (Friedrich, 2006a). Die Methode beruht auf dem Einbau von mit stabilen Isotopen (z.B. ^{13}C) markierten Substraten in die Biomasse der aktiven Mikroorganismen einer Lebensgemeinschaft und deren anschließende Identifizierung über den Nachweis der Markierung in Biomarkermolekülen wie Fettsäuren, Aminosäuren, DNA oder RNA (Fig. 1-3). In zahlreichen Übersichtsartikel werden die Konzepte der verschiedenen Methoden vorgestellt, aktuelle Anwendungen beschrieben und Ausblicke zukünftiger Entwicklungen präsentiert (Dumont and Murell, 2005; Friedrich, 2006a; Friedrich, 2006b; Madsen, 2006; Neufeld et al., 2007; Whiteley et al., 2007).

Organische Schadstoffe bestehen hauptsächlich aus Kohlenstoff und Wasserstoff. Kohlenstoff ist neben Wasserstoff und Sauerstoff eines der wichtigsten Elemente der Biosphäre und wird von allen Lebewesen für den Stoff- und Energiewechsel benötigt. Kohlenstoff besitzt zwei stabile Isotope: ^{12}C- und ^{13}C-Kohlenstoff, die natürlicherweise mit ca. 99 % und 1 % Häufigkeit in der Umwelt vorhanden sind (Tab. 1-1). Die in der Regel eine Größenordnung seltener vorkommenden schweren Isotope eines Elementes (z.B. ^{13}C-Kohlenstoff) sind zur Anwendung als Tracer geeignet.

Stabile Isotope werden als Tracer benutzt, um anhand des Verbleibs des Isotops (z.B. ^{13}C-Kohlenstoff) biologische Prozesse in der Umwelt zu untersuchen. Infolge mikrobieller Prozesse wird der isotopisch markierte Kohlenstoff von den Bakterien in ihre Biomasse

inkorporiert. Diese Anreicherung des ^{13}C-markierten Kohlenstoffs in den Biomarkermolekülen der Bakterien dient als eindeutiger Indikator für den Nachweis bestimmter biologischer Prozesse, wie zum Beispiel der Biodegradation organischer Schadstoffe.

Die Anfänge der SIP-Technologie gehen ins Jahr 1958 zurück. Zu dieser Zeit haben Meselson und Stahl erste SIP Experimente mit *Escherichia coli* und ^{15}N-markiertem Substrat (NH_4Cl) durchgeführt (Meselson and Stahl, 1958). Da die Nukleinsäuren zu einem großen Teil auch aus Kohlenstoff und Wasserstoff bestehen, wurde schnell erkannt, dass die DNA bzw. RNA ebenfalls mit den schweren Kohlenstoff und Wasserstoffisotopen markiert werden kann (Schildkraut, 1967).

Zu Beginn des 21. Jahrhunderts führten Radajewski et al. die Methode des DNA-SIP ein (Radajewski et al., 2000). Hierbei wurden Mikroorganismen zunächst in Laborexperimenten mit ^{13}C-markierten Substraten inkubiert. Der ^{13}C-Kohlenstoff der Substrate wird infolge der Assimilation in das Erbgut der aktiven Bakterien inkorporiert. Anschließend erfolgt die Extraktion der DNA aus dem Kulturansatz. Die Trennung der isotopisch schweren (^{13}C-DNA) von der isotopisch leichten DNA (^{12}C-DNA) erfolgt mithilfe der Dichtegradientenzentrifugation (Fig. 1-3). Anschließend werden die aktiven Mikroorganismen der mikrobiellen Gemeinschaft anhand der ^{13}C-markierten DNA direkt über Klonierung und Sequenzierung sowie mittels molekularbiologischer Fingerprint-Verfahren identifiziert (Friedrich, 2006b). Bereits wenig später publizierten Manefield et al. erste Anwendungen der RNA-SIP Methode (Manefield et al., 2002a; Manefield et al., 2002b).

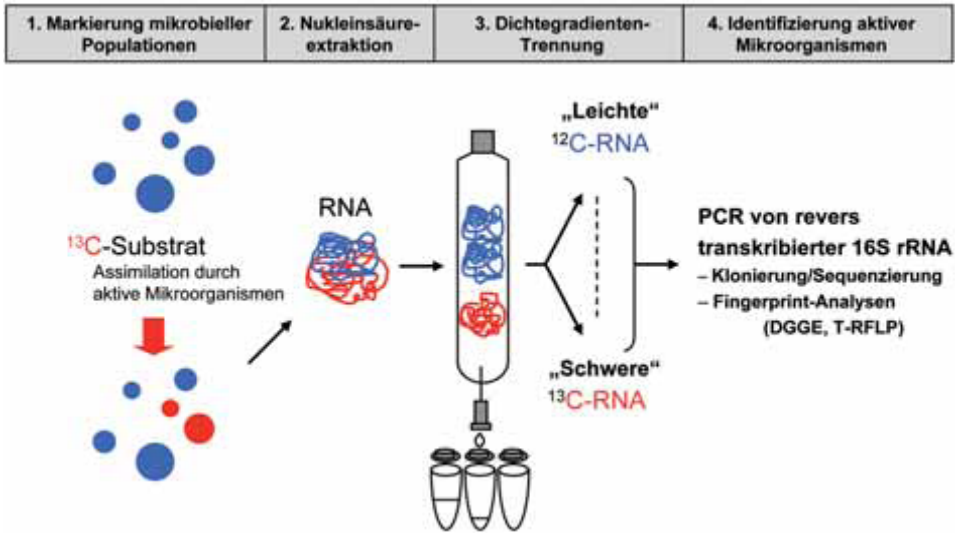

Fig. 1-3: Konzept der Stabilen Isotopenbeprobung von Nukleinsäuren am Beispiel des RNA-SIP (Friedrich, 2006a)

Die ersten DNA bzw. RNA-SIP Experimente basierten auf Untersuchungen mit C_1-Verbindungen wie ^{13}C-Methanol ($^{13}CH_3OH$), -Methan ($^{13}CH_4$) oder -Kohlendioxid ($^{13}CO_2$). Wenig später folgten dann Laborexperimente mit Aromaten und (halogenierten) Aliphaten,

bei denen die Mikroorganismen, die diese Verbindungen assimilieren können, identifiziert wurden (Friedrich, 2006b).

Eine Alternative zur stabilen Isotopenbeprobung von Nukleinsäuren stellt die Analyse der Inkorporation der ^{13}C-markierten Kohlenstoffatome in die Biomarkermoleküle wie Fettsäuren oder Aminosäuren dar. Der wesentliche Vorteil liegt hierbei in der höheren Empfindlichkeit, da die Analyse der Isotopenanreicherung direkt an den Aminosäure- bzw. Fettsäureextrakten mittels *Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry* (GC-C-IRMS) erfolgen kann (Mottram and Evershed, 2003). Mithilfe dieser Methode können bereits geringe Anreicherungen von schweren Isotopen sehr empfindlich analysiert werden. Eine Auftrennung der Biomasse in schwere und leichte Fraktion ist daher nicht erforderlich und die benötigten Mengen isotopisch markierter Substrate können minimiert werden, so dass die experimentellen Bedingungen den natürlichen Umweltbedingungen angemessen sind (Boschker and Middelburg, 2002; Boschker et al., 1998).

Fettsäuren sind wichtige Zellbestandteile, insbesondere der Zellmembranen (Doppellipidlayer). Der Anteil der Zellmembranen an der Trockenmasse einer Bakterienzelle liegt bei 8-15 % (Schlegel, 1992). Es können drei Hauptgruppen von Membranlipiden unterschieden werden: Phospho-, Glyko- und Neutrallipide. Da Phospholipide (PLFA) die lebende Biomasse charakterisieren, eignen sie sich besonders als Biomarker (Green and Scow, 2000; Kaur et al., 2005). Bestimmte Membranfettsäuren können als Biomarker benutzt werden, weil sie charakteristisch für verschiedene Organismengruppen sind (Tab. 1-2). Zum Teil kann eine weitere Unterscheidung innerhalb dieser Gruppen erfolgen. Bei den sulfatreduzierenden oder methanotrophen Mikroorganismen kann theoretisch sogar zwischen verschiedenen Gattungen differenziert werden (Tab. 1-2). Eingeschränkt kann anhand der Konzentration der Fettsäuren mithilfe von Umrechnungsfaktoren die Menge der Gesamtbiomasse beziehungsweise die Zellzahlen einer Probe abgeschätzt werden (Brinch-Iversen and King, 1990; Kaur et al., 2005; Ludvigsen et al., 1997).

Tab. 1-2: Übersicht ausgewählter Biomarker bestimmter Organismen, verändert nach (Boschker and Middelburg, 2002; Green and Scow, 2000; Kaur et al., 2005; Zelles, 1999). Für die Nomenklatur der Fettsäuren siehe Kapitel 2.4.9.

Organismus	Biomarker	Literatur
Bakterien	i15:0, a15:0, 15:0, 16:1ω5, i17:0, 17:0, 18:1ω7	(Tunlid and White, 1992)
Gram-positiv	verzweigte PLFAs (br17:0, br18:0, i17:0, i16:0, i16:1, 10Me16:0, 10Me17:0) i15:0, a15:0 einfach ungesättigte Fettsäuren <	(Harwood and Russell, 1984; O'leary and Wilkinson, 1988)

	20% (16:1ω9, 16:1ω7c, 16:1ω5, 18:1ω7c, 19:1)	
Gram-negativ	Cyclopropan-Fettsäuren (cy17:0, cy19:0) β-Hydroxy-Fettsäuren der Zellwände einfach ungesättigte Fettsäuren > 20% (16:1ω9, 16:1ω7c, 16:1ω5, 18:1ω7c, 19:1)	(Guckert et al., 1985; Parker et al., 1982; White, 1994; Wilkinson, 1988)
Sulfatreduzierer	*Desulfobacter* (10Me16:0, cy18:0(ω7,8) *Desulfovibrio* (i17:1ω7c, i15:1ω7c, i19:1ω7c) Desulfobulbus (17:1ω6, 15:1)	(Dowling et al., 1986; Edlung et al., 1985; Parker et al., 1982; Parkes and Calder, 1985; Scheuerbrandt and Bloch, 1962; Taylor and Parkes, 1983)
Methanotrophe	Typ I (16:1ω8c, 16:1ω6c) Typ II (18:1ω8c, 18:1ω8t, 18:1ω6c)	(Borjesson et al., 1998; Bowman et al., 1993; Makula, 1978; Nichols et al., 1987; Nichols et al., 1985; Sundh et al., 1995)
Aktinomyzeten	(10Me17:0, 10Me18:0)	
Pilze	mehrfach ungesättigte Fettsäuren (18:2ω6, 18:3ω6, 18:3ω3c)	(Federle, 1986; Frostegard and Bååth, 1996; Vestal and White, 1989)
Protozoa	20:2ω6, 20:3ω6, 20:4ω6	(White, 1988; White et al., 1983)
Algen	20:5ω3, 18:3ω3	

Die Anwendungen, basierend auf der Methode des PLFA-SIP, in der Umweltmikrobiologie sind zahlreich, wie in verschiedenen Übersichtsartikeln zusammengefasst wurde (Boschker and Middelburg, 2002; Evershed et al., 2006; Kreuzer-Martin, 2007; Zhang, 2002). Sehr häufig diente die Methode zur Untersuchung methanotropher Bakterien unter aeroben und anaeroben Umweltbedingungen (Blumenberg et al., 2005; Bull et al., 2000; Crossmann et al., 2005), aber auch Untersuchungen zur Aktivität der mikrobiellen Gemeinschaft im Bereich der Rhizosphäre bilden einen weiteren Anwendungsschwerpunkt dieser Methode (Butler et al., 2003; Lu et al., 2004; Treonis et al., 2004).

Der Fokus dieser Arbeit liegt allerdings auf Anwendungen, die im Zusammenhang mit Umweltschadstoffen stehen. Eine große Anzahl von ^{13}C-markierten Substraten ist bei kommerziellen Anbietern erhältlich. Die verhältnismäßig hohen Preise für ^{13}C-markierte Verbindungen limitieren jedoch maßgeblich die Anwendung im Feldmaßstab. Experimente

zum Nachweis des biologischen Abbaus organischer Schadstoffe sind daher noch verhältnismäßig selten, insbesondere unter *in situ* Bedingungen. Die ersten Untersuchungen zum Nachweis des Toluol-Abbaus erfolgten in Batchkulturen sowie in Boden-, Sediment- und Grundwasser-Mikrokosmen (Hanson et al., 1999; Mauclaire et al., 2003; Pelz et al., 2001a; Pelz et al., 2001b). Weitere Studien beschäftigten sich mit dem PAK-Abbau (Johnsen et al., 2002; Richnow et al., 1998). Ein wesentliches Problem für die Anwendung dieser Methode im Feldmaßstab bestand im Zusammenhang mit der Verfügbarkeit ausreichender Mengen ^{13}C-markierter Substrate. Dieses Problem konnte mit der Entwicklung so genannter Bio-Sep® Beads gelöst werden (Peacock et al., 2004; White et al., 2003). Bio-Sep® ist ein aktivkohlehaltiges Material, das einerseits als Substratpool für die ^{13}C-markierten Verbindungen und andererseits als Aufwuchskörper den Mikroorganismen zur Verfügung steht, die anschließend extrahiert werden können, um die Inkorporation des ^{13}C-Kohlenstoffs in den Zellbestandteilen zu analysieren. Erstmals wurden das Konzept der *in situ* Mikrokosmen mit Bio-Sep® Beads am Standort Zeitz zum Nachweis des Benzol und Toluol Abbaus im kontaminierten Grundwasser eingesetzt (Geyer et al., 2005).

Die Fettsäuren der Membranlipide waren die ersten Biomarker, die in Kombination mit stabilen Isotopenanreicherungen untersucht wurden (Boschker et al., 1998; Manefield et al., 2004). Der wesentliche Vorteil liegt in der hohen Empfindlichkeit dieser Methode, ohne dass eine Auftrennung von isotopisch leichter und schwerer Fraktion nötig wird. Daher können selbst geringe Aktivitäten detektiert werden, so dass die Mengen des bereit gestellten ^{13}C-markierten Substrats den natürlich vorkommenden Substratkonzentrationen entsprechen können. Das ist hinsichtlich Reproduzierbarkeit der natürlichen Bedingungen von Bedeutung und nicht zuletzt auch ein finanzieller Vorteil, da folglich bereits geringe Mengen der teuren ^{13}C-markierten Substrate genügen, um eine Anreicherung in den Membranlipiden nachweisen zu können. Aufgrund der extrem hohen Sensitivität ist diese Methode insbesondere für *in situ* Experimente geeignet, bei denen in der Regel nur relativ geringe Mengen an Biomasse extrahiert werden können und auch die ^{13}C-Anreicherung in den Biomarkern meist relativ gering ist.

Fettsäuren, insbesondere die PLFA, bieten zwar ein gewisses taxonomisches Potenzial (Green and Scow, 2000; Kaur et al., 2005; Zelles, 1999), aber Variationen der Gen-Sequenzen verschiedener Mikroorganismen sind deutlich höher und erlauben detailliertere Aussagen hinsichtlich den Veränderungen in der aktiven mikrobiellen Gemeinschaft. Im Gegensatz zu PLFA-SIP ist es mithilfe von DNA-SIP möglich die *in situ* Funktion verschiedener physiologischer Gruppen der mikrobiellen Gemeinschaft anhand der Kohlenstoffflüsse zu bestimmen und damit eine Charakterisierung komplexer Zusammenhänge zwischen Umweltfaktoren und physiologischer Diversität vorzunehmen (Lu et al., 2007). Allerdings sind für die Anwendung von DNA-SIP deutlich höhere ^{13}C-

Anreicherungen von > 30 % notwendig (Radajewski et al., 2003) und es bedarf mindestens zwei Zellteilungen, bis die neu synthetisierte DNA vollständig ^{13}C-markiert ist, was für eine optimale Trennung von schwerer und leichter DNA im Dichtegradienten wichtig ist (Manefield et al., 2002a; Manefield et al., 2002b).
Der Vorteil des RNA-SIP gegenüber dem DNA-SIP liegt in der größeren Sensitivität, da jedes neu synthetisierte RNA-Molekül nach Zugabe ^{13}C-markierter Substrate vollständig markiert sein kann, wodurch die RNA deutlich schneller ^{13}C-markiert wird als die DNA ohne dass es eine Zellteilung erfordert (Manefield et al., 2002a; Radajewski et al., 2003).
DNA und RNA haben ein deutlich größeres taxonomisches Potenzial als die Fettsäuren, jedoch sind relativ große Mengen Biomasse erforderlich und eine ^{13}C-Inkorporation von mindestens 30 % wird benötigt. Die resultierenden langen Inkubationszeiten können zusätzlich zum Problem des *„cross-feeding*“ innerhalb einer Nahrungskette führen. Außerdem werden größere Mengen der ^{13}C-markierten Substrate für die erfolgreiche Durchführung eines DNA- bzw. RNA-SIP Experimentes benötigt (Radajewski et al., 2003).

1.5 *In situ* Mikrokosmen

Die Methode des SIP ist geeignet, um Aktivität, Diversität und Funktionalität mikrobieller Gemeinschaften unter den verschiedensten Umweltbedingungen zu analysieren. Da die Laborergebnisse nicht ohne weiteres auf die weitaus komplexeren natürlichen Systeme übertragen werden können, besteht seit längerem das Bestreben die verschiedenen Untersuchungen möglichst unter *in situ* Bedingungen durchzuführen (Mandelbaum et al., 1997). Trotzdem gibt es bis heute nur wenige Testsysteme, die Experimente direkt unter Aquiferbedingungen ermöglichen. In einem Übersichtsartikel zu *in situ* Mikroksomen beschreiben Mandelbaum et al. (1997) zwei verschiedene *in situ* Mikrokosmen Konzepte, um einerseits in isolierten, ungestörten Bereichen des Aquifers Aussagen bezüglich physikalisch-biochemischer Parameter zu erhalten beziehungsweise um Rein- und Mischkulturen aus dem Labor direkt im Grundwasserstrom zu testen (Mandelbaum et al., 1997). In einer Studie von Hendrickx et al. (2005) wurde unkontaminiertes Aquifermaterial in einem Mesokosmos sowohl im kontaminierten als auch im unkontaminierten Bereich des Aquifers inkubiert, um *in situ* die Dynamik der sich entwickelnden mikrobiellen Gemeinschaft zu analysieren.
Um die Methode des SIP direkt im kontaminierten Aquifer zum Nachweis der Biodegradation organischer Schadstoffe anwenden zu können, wird ein Testsystem benötigt, dass einen Substratpool für die isotopisch markierten Substanzen (^{13}C-markierte Kontaminanten) zur Verfügung stellt und als Aufwuchskörper für die Grundwassermikroorganismen dient. Ziel hierbei ist es, entgegen aufwändiger und kostspieliger Tracerexperimente wie zum Beispiel Push-Pull Tests, mit weitaus geringeren Mengen ^{13}C-markierter Substanzen den direkten

Nachweis der *in situ* Biodegradation zu führen und damit perspektivisch eine effiziente Methode für die Anwendung in der Altlastenpraxis zu etablieren.

White et al. (2003) entwickelten zunächst zur Überwachung der Trinkwasserqualität einen Biofilm Sammler, bestehend aus Bio-Sep® Beads. Bei Bio-Sep® (K. L. Sublette, Tulsa, USA) handelt es sich um ein aktivkohlehaltiges Material mit besonders großer innerer Oberfläche und Poren von ca. 2-20 µm Durchmesser, was den Bakterien die Besiedlung des Kugelinneren ermöglicht. In weiteren Anwendungen wurden die Biofilm Sammler genutzt, um Veränderungen innerhalb der mikrobiellen Gemeinschaft des Grundwassers während der Zugabe verschiedener Elektronendonatoren in Push-Pull Tests zu beobachten (Istok et al., 2004; Peacock et al., 2004). Hierfür wurden die Testsysteme für mehrere Wochen in den Grundwassermessstellen inkubiert. Während dieser Zeit besiedelten die Mikroorganismen das Innere der Aufwuchskörper und bildeten Biofilme. Die spätere Extraktion der Biomasse und Analyse der Biomarker (DNA, Lipide) zeigte, dass signifikante Veränderungen innerhalb der mikrobiellen Gemeinschaft infolge der Stimulation mit verschiedenen Elektronendonatoren mithilfe dieser Art der Probenahme detektiert werden konnten.

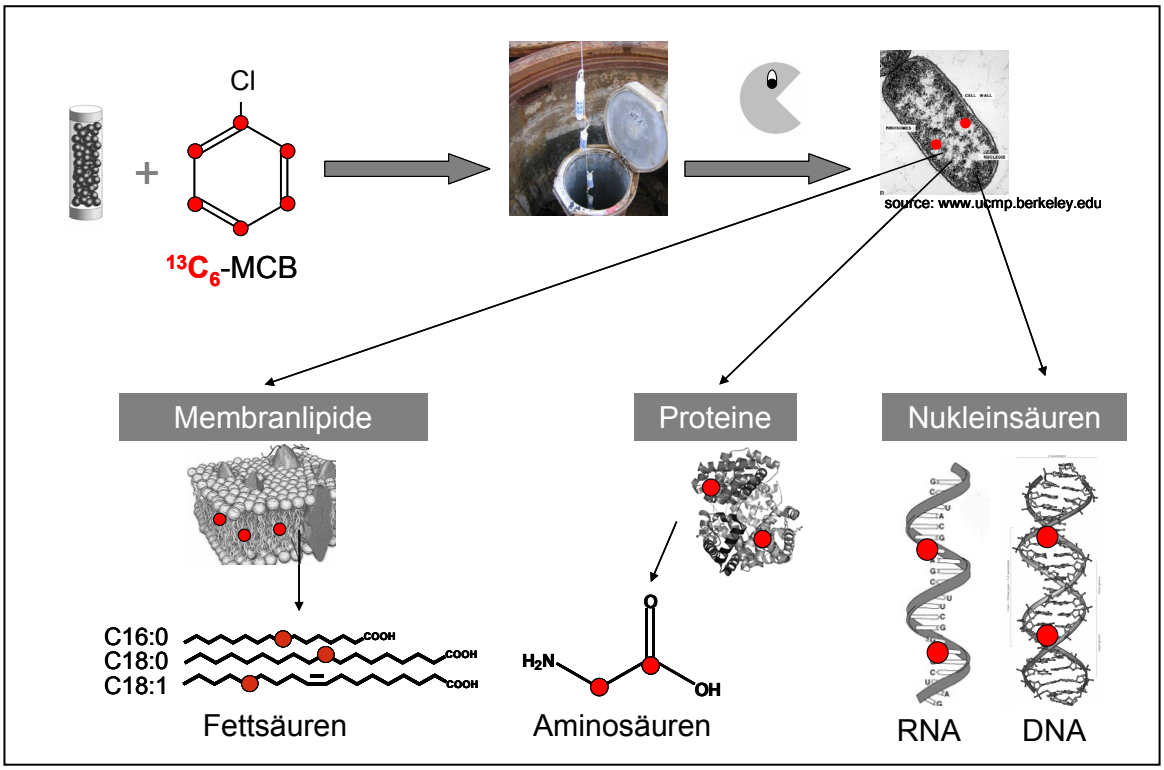

Fig. 1-4: Konzept der *in situ* Mikrokosmen (BACTRAP®) basierend auf ^{13}C-markierten Substraten zum Nachweis mikrobieller Aktivität anhand der Inkorporation der isotopischen Markierung in die Biomarkermoleküle.

In einem ersten Versuch zum Nachweis der *in situ* Biodegradation von organischen Grundwasserkontaminanten wurden Bio-Sep® Bead Sammler (BACTRAP®) zunächst im Labor mit ^{13}C-markierten Schadstoffen (^{13}C-Benzol oder ^{13}C-Toluol) beladen und anschließend in einem BTEX kontaminierten Grundwasserleiter inkubiert (Geyer et al.,

2005). Der Nachweis ^{13}C-markierter Fettsäuren sowohl auf den Toluol- als auch Benzol beaufschlagten BACTRAP®s diente als direkter Indikator für den anaeroben Abbau von Benzol und Toluol unter *in situ* Bedingungen. Damit wurde erstmalig ein Testsystem beschrieben, welches den Nachweis der *in situ* Biodegradation basierend auf der SIP-Technik direkt im kontaminierten Grundwasserleiter ermöglichte (Fig. 1-4).

1.6 Zielstellung und Inhalt der Arbeit

Die Zielsetzung für diese Arbeit war, das Testsystem der *in situ* Mikrokosmen (BACTRAP®) weiterzuentwickeln, um die Methode in der Altlastenpraxis anwenden und etablieren zu können. Hierfür war es erforderlich die Methode durch den Vergleich mit anderen Techniken zu evaluieren sowie in weiteren Feldanwendungen und mit verschiedenen Schadstoffen zu testen. Diese Arbeit beinhaltet daher Ergebnisse von mehreren Feldversuchen, die an verschiedenen Standorten und mit unterschiedlichen Schadstoffen durchgeführt wurden. Den Schwerpunkt bilden dabei Untersuchungen mit BTEX (insbesondere Benzol, Toluol) sowie Monochlorbenzol (MCB). Obwohl der anaerobe Benzolabbau unter nitrat- (Coates et al., 2001; Ulrich and Edwards, 2003), eisen (III)- (Anderson et al., 1998; Cozzarelli et al., 1990; Lovley, 2000; Lovley et al., 1989; Lovley et al., 1994; Lovley et al., 1996) und sulfatreduzierenden (Anderson and Lovley, 2000; Lovley, 1995; Lovley, 2000; Wiedemeier et al., 1999) sowie methanogenen (Bekins et al., 2001; Grbic-Galic and Vogel, 1987) Bedingungen bereits seit mehreren Jahren bekannt ist, konnten die am Abbau beteiligten Mikroorganismen bisher nicht isoliert werden. MCB galt sogar bis vor kurzem unter anoxischen Bedingungen als persistente, nicht abbaubare Verbindung. Ergebnisse dieser Dissertation (Kapitel 5 bis 7) lieferten den Nachweis, dass unter anaeroben Bedingungen der Abbau von MCB erfolgen kann. MCB wurde hierbei als Modellverbindung für sehr persistente Substrate betrachtet.

Die Arbeitsziele dieser Promotionsarbeit lauteten:

- Validierung des Testsystems zur Beurteilung der *in situ* Biodegradation kontaminierter Aquifere mithilfe alternativer Methoden
- Anwendung der *in situ* Mikroksomen an verschiedenen Feldstandorten und für unterschiedliche Schadstoffe
- Weiterentwicklung des Testsystems zur Analyse mikrobieller Konsortien und Rekonstruktion von Abbauwegen
- Charakterisierung des intrinsischen Abbaupotenzials persistenter Schadstoffe an komplexen Standorten

- Implementierung in die Altlastenpraxis als Nachweismethode zur Erfassung der *in situ* Biodegradation im Kontext von *Natural Attenuation* als Standortsicherungsmaßnahme

Die vorliegende Arbeit ist eine kumulativ verfasste Dissertationsschrift, deren Hauptteil sich aus sechs Veröffentlichungen zusammensetzt. Davon wurden drei Artikel als Erstautor verfasst, von denen bereits zwei im Jahr 2006 in den Fachzeitschriften *Grundwasser* und *Organic Geochemistry* veröffentlicht werden konnten. Der dritte Artikel wurde 2008 zur Veröffentlichung bei *Environmental Science and Technology* eingereicht. Bei den anderen Publikationen hat die Doktorandin als Co-Autorin maßgeblich mitgewirkt und war sowohl bei Feldversuchen und Laborarbeiten, als auch bei der Erstellung der Manuskripte wesentlich beteiligt. Die Labor- und Feldarbeiten wurden teilweise von Diplomanden und Praktikanten unterstützt, wobei die Autorin aktiv und verantwortlich bei der Betreuung der Arbeiten mitgewirkt hat.

Die Dissertation beginnt nach einer allgemeinen, übergreifenden Einleitung (Kapitel 1) im Hauptteil mit einer Gegenüberstellung geochemischer und isotopenchemischer Methoden zur Charakterisierung des anaeroben Benzolabbaus im Rahmen eines Multilevel-Experimentes (Kapitel 2). Das Kapitel 2, 2006 in der Fachzeitschrift *Grundwasser* (Band 11(4), S. 247-258) veröffentlich, dient des Weiteren als umfangreiche Methodenbeschreibung zur Herstellung, Installation und Extraktion der *in situ* Mikrokosmen. Der Artikel wurde in einer deutschsprachigen Fachzeitschrift veröffentlicht, um auf diesem Weg möglichst eine Vielzahl der deutschen Sanierungspflichtigen, Behörden und Ingenieurbüros aus dem angewandten Bereich zu erreichen.

Kapitel 3, 2006 in *Organic Geochemistry* (Band 37(10), S. 1394-1410) publiziert, präsentiert weitere Beispiele zum Nachweis der *in situ* Biodegradation von Benzol und Toluol im hoch- und niedrig kontaminierten Grundwasser an zwei verschiedenen Feldstandorten. Außerdem wird der Frage nachgegangen, ob *in situ* Mikrokosmen geeignet sind, Abbauwege anhand von Metaboliten, die sich auf dem Testsystem anreichern, rekonstruieren und die mikrobielle Gemeinschaft des Grundwassers charakterisieren zu können. Hierfür wurde alternativ zur Analyse der Fettsäureprofile die Methode des *Single Strand Conformation Polymorphism* (SSCP) gewählt. Die verschiedenen Untersuchungsergebnisse (Geochemie, *in situ* Mikrokosmen, Molekularbiologie) dienten einer Verifizierung der Methode der BACTRAP®s.

Kapitel 4, 2006 als Übersichtsartikel in *Engineering Life Science* (6(3), S. 234-251) veröffentlicht, dokumentiert den gegenwärtigen Stand des Wissens über Verfahren zur Beurteilung der *in situ* Biodegradation im kontaminierten Grundwasser. Es werden die aktuellen Ergebnisse und Fortschritte der Methode der Stabilen Isotopenfraktionierungsanalyse (SIFA) sowie der *in situ* Mikrokosmen vorgestellt und diskutiert. Außerdem wird der Frage nachgegangen, ob die mikrobielle Gemeinschaft, die

das *in situ* Testsystem besiedelt, tatsächlich die Mikroflora des Aquifers repräsentiert, in dem die Biomasse des Grundwassers und des Testsystems mittels SSCP verglichen wurden. Ziel dieser Betrachtung ist es zu prüfen, ob die Ergebnisse der *in situ* Mikrokosmen auf den Grundwasserleiter übertragbar sind, eine wichtige Voraussetzung für die Erfassung des *in situ* Abbaupotenzials an kontaminierten Standorten.

Einen Schwerpunkt dieser Dissertation stellen Untersuchungen zum Nachweis des anaeroben Abbaus von Monochlorbenzol dar. Die diesbezüglichen Ergebnisse sind in den Kapitel 4, 5, 6 und 7 aufgeführt. Kapitel 5, 2007 in *Environmental Science and Technology* (41, S. 3836-3842) publiziert, befasst sich im Wesentlichen mit dem sensitiven Nachweis des anaeroben MCB Abbaus in Tracerversuchen mit ^{13}C-markiertem MCB in *in situ* und *ex situ* Mikrokosmen.

Kapitel 6, 2008 zur Veröffentlichung bei *Environmental Science and Technology* eingereicht, zeigt die Entwicklung eines integrativen Untersuchungskonzepts für die Beurteilung des Natural Attenuation Potenzials komplexer Standorte, die durch multiple, schwer abbaubare Schadstoffe wie chlorierte Benzole, sequentielle Abbauwege und heterogene Aquiferbedingungen charakterisiert sind. Um plausible Aussagen bezüglich der biogeochemischen Prozesse eines Untersuchungsstandortes zu erhalten, müssen neuartige Untersuchungsmethoden wie CSIA und BACTRAP® sowie statistische Auswertungsmethoden miteinander kombiniert werden.

Mit der Frage nach weiteren Anwendungsfeldern für den Einsatz von *in situ* Mikrokosmen beschäftigt sich Kapitel 7, dass 2007 in *Environmental Pollution* (148, S. 428-437) veröffentlicht wurde. Es handelt sich ebenfalls um eine Studie zum MCB Abbau, allerdings in einer Pflanzenkläranlage. Zielsetzung war es die Abbauleistung der Pflanzenkläranlage für MCB zu evaluieren. Erstmalig wurden hierfür *in situ* Mikrokosmen direkt im Sediment inkubiert.

Der Hauptteil der Dissertation endet mit einer kritischen Zusammenfassung und Diskussion aller Ergebnisse in Kapitel 8.

2 Analyse des anaeroben Benzolabbaus: Vergleich von *In situ* Mikrokosmen, Elektronenakzeptorbilanzen und Isotopenfraktionierungsprozessen

Nicole Stelzer, Anko Fischer, Matthias Kästner, Hans-H. Richnow

(Dieses Kapitel wurde in der Zeitschrift Grundwasser Band 11(4), S. 247-258 veröffentlicht)

2.1 Kurzfassung

Geochemische und isotopenchemische Methoden wurden zur Charakterisierung des anaeroben Benzolabbaus am Standort eines ehemaligen Hydrierwerkes (Zeitz, Deutschland) genutzt. Im Abstrom des Schadenszentrums wurde hierfür die vertikale Struktur der Benzolfahne in verschiedenen Tiefen untersucht. Durch Einsatz von [$^{13}C_6$] Benzol in *in situ* Mikrokosmen konnte anhand der Transformation des markierten Kohlenstoffs in die Biomasse eindeutig das Abbaupotenzial nachgewiesen werden. Das Fettsäurespektrum sowie deren Isotopensignaturen deuten auf eine Besiedlung durch komplexe mikrobielle Gemeinschaften hin, die in unterschiedlicher Weise am Benzolabbau beteiligt sind. Die Sulfatsenke in der Schadstofffahne deutet auf überwiegend sulfatreduzierende Abbaubedingungen mit einem Abbaupotenzial von etwa 1,7 mmol L^{-1} Benzol hin. Anhand der Isotopensignaturen und Konzentrationen des DIC wurde in der Schadstofffahne ein Abbau von 2,2 mmol L^{-1} Benzol abgeschätzt. Eine Berechnung des Benzolabbaus anhand der Isotopenfraktionierungsmethode ergibt einen Abbau von etwa 3,0 mmol L^{-1} auf dem Fließweg. Die quantitativen geochemischen und isotopenchemischen Abschätzungen liegen in der gleichen Größenordnung und zeigen einen signifikanten Benzolabbau im oberen Aquifer.

2.2 Abstract

Geochemical and isotope chemical methods were applied to assess the *in situ* biodegradation of benzene in a shallow aquifer (Zeitz, Germany). The vertical structure of the plume was investigated in a multi level sampling approach. Benzene degradation was investigated using *in situ* microcosms incubated with [$^{13}C_6$]-labelled benzene. The transformation of ^{13}C into fatty acids proved biodegradation and indicated that a complex microbial community is colonizing the *in situ* microcosms.

The sink of sulphate in the course of the plume indicated sulphate reducing conditions which may account for the oxidation of 1.7 mmol L^{-1} benzene. The concentration and isotope composition of dissolved inorganic carbon indicated a degradation of 2.2 mmol L^{-1} benzene within the plume. The isotope composition of benzene suggested an average degradation of

3.0 mmol L^{-1} benzene on the groundwater flow path. The various approaches to assess the benzene degradation were in the same order and illustrated significant benzene degradation in the anaerobic aquifer.

2.3 Einleitung

Benzol, Toluol, Ethylbenzol und Xylole (BTEX) sind häufige Grundwasserkontaminanten, die eine Gefahr für Ökosysteme und die menschliche Gesundheit darstellen. In einem verunreinigten Aquifer wird der Verbleib dieser Verbindungen durch abiotische (Advektion, Dispersion, Verflüchtigung, Sorption) und biologische Prozesse beeinflusst. Eine nachhaltige Reduzierung der Schadstoffmenge erfolgt allerdings hauptsächlich durch den mikrobiellen Abbau. Bei der Anwendung von Sanierungsstrategien wie *Monitored Natural Attenuation* (MNA) und *Enhanced Natural Attenuation* (ENA) kommt somit dem mikrobiellen Schadstoffabbau eine besondere Bedeutung zu. Es werden daher geeignete Methoden benötigt, um das natürliche Selbstreinigungspotenzial eines kontaminierten Grundwasserleiters bestimmen zu können.

Eine Möglichkeit zur Untersuchung des *in situ* Abbaus von Schadstoffen ist die Nutzung der substratspezifischen Isotopenfraktionierung (Meckenstock et al., 2004a). Diese Methode beruht darauf, dass Bakterien infolge des mikrobiellen Schadstoffabbaus das natürliche Isotopenverhältnis (z.B. $^{13}C/^{12}C$) eines Substrates verändern. Die bevorzugte Verwertung der leichten Isotopomere einer Kontaminante führt infolge der mikrobiellen Umsetzung zu einer Anreicherung der schweren Isotopomere in der residualen Schadstofffraktion, was mit Hilfe der Gaschromatographie-Isotopenverhältnis-Massenspektroskopie (GC-IRMS) analytisch nachgewiesen werden kann.

Zur Abschätzung der Biodegradation in kontaminierten Grundwasserleitern werden verschiedene Verfahren angewendet, die häufig auf der Kultivierung von Mikroorganismen im Labor beruhen. Der anaerobe Schadstoffabbau ist in der Regel ein sehr langsam ablaufender Prozess und viele der am Abbau beteiligten Mikroorganismen sind bisher noch nicht oder nur eingeschränkt kultivierbar. Labortestverfahren erscheinen daher nur begrenzt geeignet zu sein, um die realen, sehr komplexen Vorgänge und anoxischen Milieubedingungen des fließenden Grundwassers abzubilden. Darüber hinaus sind anaerobe Abbauversuche sehr langwierig und generell mit einem relativ großen Zeitaufwand verbunden.

Hieraus resultiert ein großer Bedarf an der Entwicklung geeigneter *in situ* Methoden zur Bestimmung der im Grundwasserleiter stattfindenden Biodegradation, insbesondere für anoxische Milieubedingungen.

Der mikrobielle Abbau aller BTEX-Verbindungen konnte unter oxischen und anoxischen Bedingungen nachgewiesen werden. Aufgrund der Sauerstofflimitation findet in

kontaminierten Grundwasserleitern meist eine anaerobe Biodegradation der monoaromatischen Kohlenwasserstoffe statt (Cerniglia, 1984; Chakraborty and Coates, 2004; Gibson and Subramanian, 1984; Wiedemeier et al., 1999). Dabei ist Benzol im Vergleich zu Toluol, Ethylbenzol und Xylolen oft schwerer abbaubar, so dass folglich bis heute nur wenige anaerob benzolabbauende Konsortien und eine Reinkultur isoliert wurden (Chakraborty and Coates, 2004; Coates et al., 2001; Wiedemeier et al., 1999). Das begründet, warum in Laborstudien der anaerobe Benzolabbau bisher nur relativ selten eindeutig nachgewiesen werden konnte und sich in Kulturversuchen nur schwer reproduzieren lässt.

Tracerexperimente mit isotopisch markierten Substanzen stellen eine alternative Methode zur Untersuchung der Biodegradation im Grundwasser dar. Radioaktiv markierte Substanzen (^{14}C) sollten aufgrund ökologischer Risiken nicht in offenen Systemen eingesetzt werden. Ersatzweise dienen stabile Isotope (D, ^{13}C) als Tracer. Während der Assimilation wird der isotopisch markierte Kohlenstoff von den Mikroorganismen zur Synthese von Biomasse verwendet. Durch den Einsatz von ^{13}C-markierten Substraten kann der mikrobielle Schadstoffabbau anhand der Anreicherung des ^{13}C-markierten Kohlenstoffs in verschiedenen Zellkomponenten nachgewiesen werden. Dabei stehen als Zellmembranbestandteile häufig Phospholipid-Fettsäuren (**p**hospho**l**ipid-derived **f**atty **a**cids, PLFA) im Mittelpunkt der Untersuchungen (Abraham et al., 1998; Boschker and Middelburg, 2002; Taylor and Parkes, 1983). Es sind nur wenige Arbeiten zur Nutzung ^{13}C-markierter Substrate für die Bestimmung des mikrobiellen Schadstoffabbaus anhand der ^{13}C-Anreicherung in PLFA bekannt. Die Anreicherung von ^{13}C in verschiedenen PLFA diente zum Nachweis des Toluolabbaus. Dabei wurde Aquifermaterial in Labormikrokosmen mit [^{13}C]-α-Toluol als Substrat inkubiert, die Transformation des Isotopensignals in die Biomasse nachgewiesen, und die am anaeroben Toluolabbau beteiligten Mikroorganismen identifiziert (Pelz et al., 2001a; Pelz et al., 2001b). In ähnlichen Studien mit [$^{13}C_6$]-markiertem Toluol konnten anhand des Einbaus von ^{13}C in PLFA die mikrobiellen Populationen in aeroben Bodenmikrokosmen identifiziert werden (Hanson et al., 1999). In einem *"Push & Pull"*-Tracerexperiment mit [2-^{13}C]-Acetat als Substrat und Nitrat als Elektronenakzeptor ist anhand der Transformation der ^{13}C-Markierung in die verschiedenen PLFA die mikrobielle Abbauaktivität im Aquifer nachgewiesen worden (Pombo et al., 2002). Demgegenüber wurden spezielle *in situ* Mikrokosmen (BACTRAP®) entwickelt, die mit ^{13}C-markierten Testsubstanzen beladen und direkt *in situ* im Aquifer inkubiert werden können. Anhand der Transformation der isotopischen Markierung in die Biomasse (Membranfettsäuren) kann der Schadstoffabbau nachgewiesen werden. Zur Erprobung dieses Testsystems wurden verschiedene Experimente durchgeführt (Büning et al., 2005; Geyer et al., 2005).

Die vorliegende Arbeit stellt die Ergebnisse eines Feldversuches vor, bei dem BACTRAPs zur Analyse des Benzolabbaus genutzt wurden. Dabei sind die Mikrokosmen mit [$^{13}C_6$] Benzol als Substrat beladen und mit Hilfe eines Multilevel-Packersystems (MLPS) in acht verschiedenen Tiefen einer Grundwassermessstelle eines BTEX-kontaminierten Aquifers während 51 Tagen inkubiert worden. Der Einbau der ^{13}C-Atome aus dem Benzol in mikrobielle Fettsäuren wurde im Anschluss mittels GC-IRMS nachgewiesen und als Beweis für den *in situ* Benzolabbau genutzt. Hydrogeo- und isotopenchemische Parameter dienten zur Charakterisierung des Abbaumilieus. Darüber hinaus wurde der *in situ* Benzolabbau mit Hilfe der Isotopenfraktionierungsmethode und anhand von Elektronenbilanzen abgeschätzt. Die Diskussion der Ergebnisse erfolgte im Hinblick auf den Einsatz verschiedener Methoden zur Kennzeichnung des *Natural Attenuation* Potenzials.

2.4 Material und Methoden

2.4.1 Isotopen markierte Substanzen und Chemikalien

Es wurden ausschließlich Chemikalien in analytischer Qualität (p. A.) verwendet. Isotopisch markiertes [$^{13}C_6$] Benzol wurde von Sigma-Aldrich (St. Louis, USA) mit einer chemischen Reinheit von 99 % bezogen.

2.4.2 Standortbeschreibung

Bei dem Untersuchungsstandort handelt es sich um einen BTEX-Schadensfall im Bereich eines ehemaligen Hydrierwerkes bei Zeitz (Sachsen-Anhalt, Deutschland). Am Standort sind im Wesentlichen zwei Grundwasserleiter ausgebildet, die durch einen eozänen Braunkohle-führenden Ton-Schluff-Komplex voneinander getrennt sind, jedoch lokal teilweise infolge Erosion des Stauerkomplexes in direktem hydraulischem Kontakt stehen. Die Geschichte, Hydrogeologie und Geochemie des Standortes wurden ausführlich beschrieben (Dahmke et al., 2004; Fischer et al., 2004; Gödeke et al., 2004a; Gödeke et al., 2004b; Schirmer et al., 2006; Vieth et al., 2001; Vieth et al., 2005). Benzol stellt mit einem Anteil von mehr als 99 % die Hauptkontaminante dar, wobei im Zentrum der Schadstofffahne Konzentrationen von > 1000 mg L^{-1} bestimmt wurden. Der mikrobielle BTEX-Abbau ist überwiegend durch anaerobe Prozesse wie Sulfatreduktion geprägt (Fischer et al., 2004; Vieth et al., 2005).

Die hier beschriebenen Untersuchungen wurden im oberen, ca. 2-10 m mächtigen jungtertiären und quartären Aquifer (GWL I) durchgeführt, der hauptsächlich aus Terrassenschottern der Weißen Elster besteht. Die vertikale Struktur der Kontaminationsfahne wurde im Bereich der Grundwassermessstelle Saf ZZ 23/00, die sich im nördlichen Abstrom des Kontaminationsherdes befindet, untersucht (Fig. 2-1).

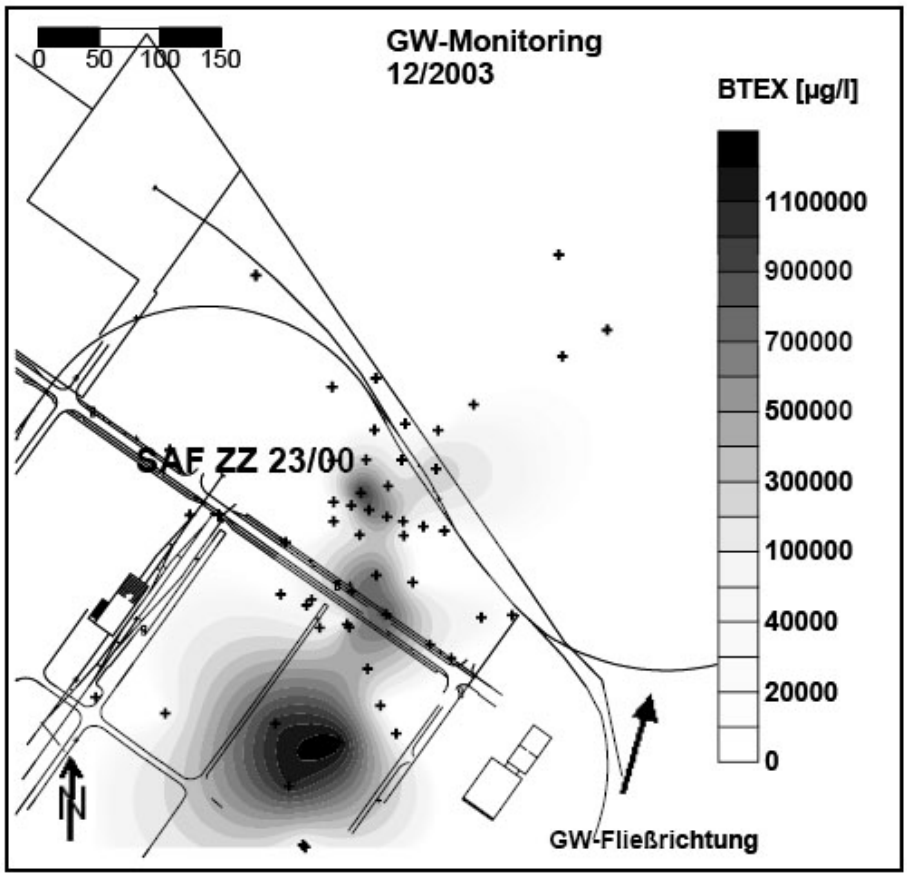

Fig. 2-1: Verteilung der BTEX-Konzentrationen (µg L^{-1}) im oberen Aquifer im Bereich des ehemaligen Hydrierwerkes bei Zeitz (Sachsen-Anhalt).

2.4.3 Tiefenorientierte Grundwasserbeprobung

Die tiefenorientierte Grundwasserbeprobung wurde mit Hilfe eines Multilevel-Packersystems (MLPS) durchgeführt (Schirmer et al., 1995). Die Förderung des Grundwassers erfolgte dabei mit pneumatisch regulierbaren Miniatur-Doppelventil-Pumpen (Innovative Messtechnik Weiß), die eine gleichmäßige und blasenfreie Probennahme gewährleisteten. Bei geringer Förderleistung (ca. 100 ml min^{-1}) sowie einem Abstand zwischen den einzelnen Pumpen von etwa einem Meter werden bei der Entnahme der Grundwasserproben vertikale hydrogeochemischen Gradienten nicht durch Vermischungseffekte beeinflusst. Für die Erzeugung des benötigten Pumpendruckes wurde Stickstoff aus einer Druckflasche benutzt, um Sauerstoffeintrag während der Probenahme auszuschließen. Mit Ausnahme des Horizontes in 9,40 m Tiefe konnte aus allen verbleibenden 7 Tiefen das benötigte Probenvolumen für die Analysen entnommen werden (Fig. 2-2). Die Grundwasserproben wurden bis zur weiteren Analyse bei 4 °C kühl und dunkel gelagert.

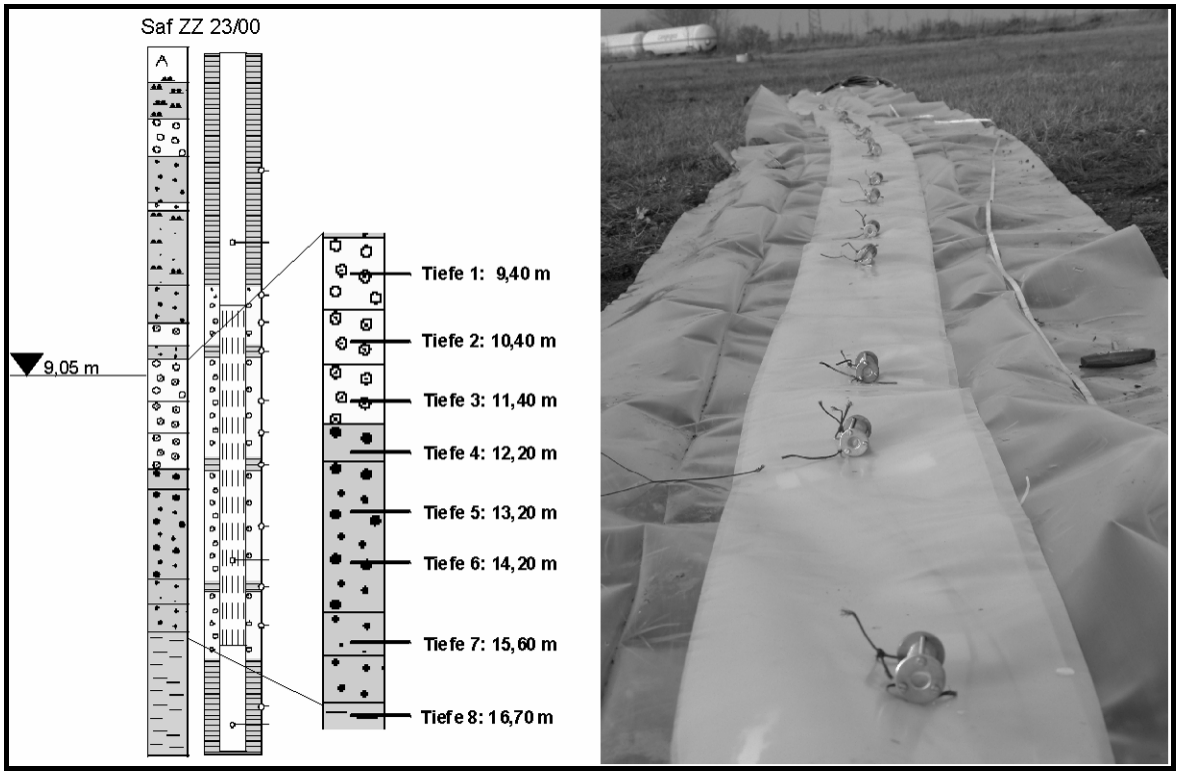

Fig. 2-2: Links, Schichtenprofil und Ausbauplan der Grundwassermessstelle Saf ZZ 23/00. Tiefe 1 bis 8 bezeichnet die Position der Miniatur-Doppelventilpumpen und BACTRAPs. Rechts, Foto des MLPS mit Edelstahlkäfigen für BACTRAPs vor dem Einbau in die Messstelle.

2.4.4 Analytik der GW-Proben

Die Benzolkonzentrationen sind gaschromatographisch nach DIN 38407-F9-1 bestimmt worden. Die ionenchromatographische Analyse der Sulfat- und Nitrat-Konzentrationen basiert auf DIN EN ISO 10304-1 D19. Die Bestimmung der Konzentration des gelösten anorganischen Kohlenstoffs (DIC) erfolgte nach DEV D8, der Eisen(II)-Konzentrationen nach DIN 38406-E1-1 und der Eisen(III)- sowie Mangan-Konzentrationen nach DIN EN ISO 11885-E22.

Zur Bestimmung der Kohlenstoffisotopensignaturen des Benzols ($\delta^{13}C_{Benzol}$), des Methans ($\delta^{13}C_{CH4}$) und des DIC ($\delta^{13}C_{DIC}$) sind erprobte Methoden verwendet worden (Fischer et al., 2004; Vieth et al., 2005).

2.4.5 Herstellung der BACTRAPs

Bei der Herstellung der BACTRAPs dienten Bio-Sep®-Kugeln (K. L. Sublette; Universität Tulsa, USA) als Trägermaterial für die Testsubstanzen [^{12}C]- und [$^{13}C_6$] Benzol und als Aufwuchskörper für die Mikroorganismen. Dabei handelt es sich um Kugeln mit 2-3 Millimeter Durchmesser, bestehend aus 75 % pulverisierter Aktivkohle eingebettet in einer Matrix aus thermostabilem Nomex®. Die Oberfläche der Kugeln weist Poren mit einem

Durchmesser von 2-10 µm auf. Bei einer Dichte von ca. 0,16 g cm^{-3} zeichnen sich die Kugeln besonders durch ihre hohe innere Oberfläche von > 600 m^2 g^{-1} Bio-Sep®-Material aus (White et al., 2003). Die Kugeln wurden zur Entfernung residualer organischer Kohlenstoffverbindungen bei 300 °C im Muffelofen für ca. 3 Stunden ausgeheizt.

Für die Herstellung der BACTRAPs ist ein Teflonrohr (VWR Darmstadt, Deutschland) mit 10 Millimeter Durchmesser in jeweils 40 Millimeter lange Stücke zerschnitten und anschließend perforiert worden, um einen Grundwasserdurchfluss gewährleisten zu können (Fig. 2-3). Pro BACTRAP wurden jeweils 0,4 g Bio-Sep® genutzt. Ein Glaswollestopfen am oberen und unteren Ende verhinderte das Herausfallen des Bio-Sep®-Materials.

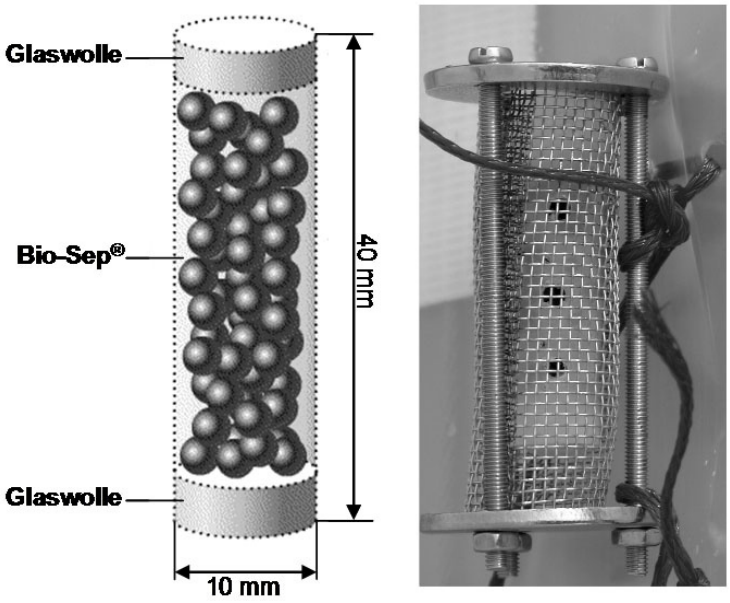

Fig. 2-3: Schematischer Aufbau eines BACTRAP (links) und Foto der Befestigung des Edelstahlkäfiges mit BACTRAP an der Packermembran (rechts).

2.4.6 Beladung der BACTRAPs

Die BACTRAPs wurden im Autoklav sterilisiert und hydratisiert. Im Anschluss erfolgte die Beladung der Mikrokosmen mit [^{12}C]- bzw. [$^{13}C_6$] Benzol. BACTRAPs ohne Benzolaufschlag dienten als Kontrolle zur Analyse der mikrobiellen Besiedlung ohne zusätzliches Substrat.

Die BACTRAPs wurden in verschließbare Glasgefäße überführt und jeweils mit etwa 76 mg Benzol pro Gramm Bio-Sep® bei einem Unterdruck von ca. 60 mbar beladen. Um einen Gleichgewichtszustand in der Gasphase herzustellen und eine einheitliche Beladung der Aktivkohle mit dem Benzol gewährleisten zu können, verblieben die BACTRAPs zur Inkubation bei Raumtemperatur für mindestens 48 Stunden in dem verschlossenen Gefäß. Die Beladung ist mit einer Standardabweichung von ± 5 % reproduzierbar (Geyer et al., 2005). Die BACTRAPs wurden im Anschluss in einer Anaerobbox mit sterilem, anaeroben Wasser überschichtet, kurz evakuiert und dann unter anoxischer Atmosphäre entspannt.

Dadurch füllte sich ein Teil des Porenraums der Kugeln mit sauerstofffreiem Wasser und der Kontakt mit Luftsauerstoff beim Einbau der BACTRAPs wurde vermieden. Die BACTRAPs wurden in diesem Wasser bis zur Installation in den Brunnen gelagert.

2.4.7 Einbau in die Grundwassermessstelle

Die BACTRAPs wurden mittels MLPS in acht Tiefen der Messstelle Saf ZZ 23/00 (Fig. 2-2) eingebracht. Um eine gleichmäßige Durchströmung der BACTRAPs mit dem Grundwasser zu gewährleisten, wurden sie in Edelstahlkäfigen eingefasst (Fig. 2-3). Die Edelstahlkäfige dienten dabei als Abstandhalter um direkte Kontakte mit dem Pegelrohr sowie der Packermembran zu vermeiden.

2.4.8 Probenaufbereitung

Nach einer Expositionszeit von 51 Tagen wurden die Mikrokosmen aus der Messstelle entnommen und zur Konservierung bei -15 °C gelagert.
Die Extraktion der Fettsäuren erfolgte nach einer modifizierten Methode von Bligh und Dyer (1959). Dabei wurden 2 ml Methanol und Dichlormethan im Verhältnis 1:3 zugegeben. Für die Herstellung der Fettsäuremethylester (FAME) dienten 300 µl eines Derivatisierungsreagenz aus Chlortrimethylsilan und wasserfreiem Methanol im Verhältnis 1:8. Die Veresterung erfolgte bei einer Temperatur von 70 °C und einer Reaktionsdauer von zwei Stunden. Die FAME wurden in Hexan gelöst und anschließend mittels GC-MS bzw. GC-IRMS analysiert.

2.4.9 Identifikation der Fettsäuremethylester (FAME) mittels GC-MS

Die Bestimmung der FAME erfolgte mit einem Gaschromatographen (HP 6890 Series, Agilent Technology, USA) mit gekoppeltem Massenspektrometer (HP 5973 Mass Selective Detector, Agilent Technologie, USA). Zur gaschromatographischen Trennung der FAME wurde eine BPX 5-Säule (30m x 0,32 mm x 0.25 µm FD; Agilent Technologie, USA) eingesetzt. Das Temperaturprogramm beginnt bei 120 °C. Nach 4 Minuten steigt die Temperatur mit 4 °C min^{-1} auf 250 °C und schließlich mit 20 °C min^{-1} auf 300 °C, die abschließend 10 Minuten konstant gehalten wurden. Die FAME sind mit Hilfe des *Bacterial Acid Methyl Ester*-Standards (BAME, Supelco) durch den Vergleich der Retentionszeiten und Massenspektren identifiziert und auf ihre Reinheit geprüft worden. Die Fettsäuren werden nach folgender Nomenklatur benannt: A:BωC, wobei A die Anzahl der Kohlenstoffatome, B die Anzahl der Doppelbindungen und C die Entfernung der nächsten Doppelbindung vom aliphatischen Ende des Moleküls repräsentiert (ω-Nomenklatur). Durch die Vorsilben *iso* (i-) und *anteiso* (a-) wird die Position der Methylverzweigung gekennzeichnet.

2.4.10 Analyse der Kohlenstoffisotopenverhältnisse der FAME mittels GC-C-IRMS

Zur Analyse der Isotopenverhältnisse der FAME wurde ein *Gas Chromatography-Combustion Isotope-Ratio-Monitoring Mass Spectrometer* System (GC-C-IRMS) verwendet. Das System besteht aus einem Gaschromatograph (HP 6890 Series, Agilent Technology, USA), der an einen Verbrennungsofen, ein GC-C-III-Interface (Combustion III, Finnigan MAT, Deutschland), eine Wasserfalle (Nafion®-Membran) und ein Massenspektrometer (IRMS MAT 252, Finnigan MAT, Deutschland) gekoppelt ist. Eine Beschreibung zur verwendeten Gerätekonfiguration geben Richnow et al. (2003a).
Zur Auftrennung der FAME wurde eine BPX 5-Säule (50m x 0,32 mm x 0,5 µm FD; SGE GmbH, Deutschland) mit folgendem Temperaturprogramm verwendet: eine Minute 70 °C isotherm, 20 °C min^{-1} auf 170 °C, 2 °C min^{-1} auf 280 °C und mit 20 °C min^{-1} auf 300 °C, die für 5 Minuten isotherm gehalten wurden.

2.4.11 Berechnungen

Die Isotopensignaturen werden als δ-Notation [‰] relativ zu dem internationalen VPDB-Standard (**V**ienna **P**ee**D**ee **B**elemnite mit $^{13}C/^{12}C$ = 0,0112372) (Clark and Fritz, 1997; Hoefs, 1997) angegeben (Gleichung 1-1).

Gleichung 1-1:
$$\delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{Probe} - \left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}}{\left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}} \times 1000$$

Während der Methylierung der Fettsäuren (FA) zu Fettsäuremethylestern (FAME) wird ein zusätzliches Kohlenstoffatom in das Produkt eingefügt, so dass die ursprüngliche Isotopensignatur aller FA konstant verändert wird. Die gemessenen $\delta^{13}C$-Werte der FAME wurden deshalb nach der Gleichung 1-2 korrigiert, um die Isotopenverhältnisse der Fettsäuren ($\delta^{13}C_{FA}$) zu ermitteln (Abraham et al., 1998).

Gleichung 1-2 :
$$\delta^{13}C_{FA} = \frac{\left[(C_n + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}\right]}{C_n}$$

C_n entspricht der Anzahl der Kohlenstoffatome in den Fettsäuren, $\delta^{13}C_{FAME}$ ist die Isotopensignatur der Fettsäuremethylester (FAME) und $\delta^{13}C_{MeOH}$ die Isotopensignatur des für die Methylierung benutzten Methanols [-38,15 ‰].

Mit der Rayleigh-Gleichung (Gleichung 1-3) wird die Beziehung zwischen der Konzentrationsänderung der mikrobiell umgesetzten Substanz in Verbindung mit einer Änderung der Isotopensignatur durch den Isotopenfraktionierungsfaktor α dargestellt (Hoefs, 1997).

Gleichung 1-3: $$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\left(\frac{1}{\alpha}-1\right)}$$

R_0 und R_t geben die Isotopenzusammensetzung (Gleichung 1-4), C_t und C_0 die Konzentration des Substrats zum Zeitpunkt t und 0 der mikrobiellen Umsetzung an.

Gleichung 1-4: $$\frac{R_t}{R_0} = \frac{\delta_t{}^{13}C \times 1000}{\delta_0{}^{13}C \times 1000}$$

Der prozentuale biologische Abbau der residualen Schadstofffraktion wird nach Gleichung 1-5 berechnet (Meckenstock et al., 2002). Dabei wird (C_t/C_0) durch Umstellen der Rayleigh-Gleichung ersetzt (Gleichung 1-6), so dass der prozentuale biologische Abbau unter Verwendung von Isotopenwerten quantifiziert werden kann.

Gleichung 1-5: $$B[\%] = \left(1 - \frac{C_t}{C_0}\right) * 100$$

Gleichung 1-6 : $$B[\%] = \left[1 - \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\frac{1}{\alpha}-1}\right)}\right] \times 100$$

Die Veränderung der Kohlenstoffisotopensignatur und der Konzentration des gelösten anorganischen Kohlenstoffs (DIC) innerhalb einer Schadstofffahne dienen als biogeochemischer Indikator und können zur Abschätzung der Schadstoffmineralisierung nach Gleichung 1-7 verwendet werden (Fischer et al., 2004).

Gleichung 1-7 $$A \times \delta^{13}C_{DIC(GA)} + (1 - A) \times \delta^{13}C_{DIC(Q)} = \delta^{13}C_{DIC(M)}$$

Dabei bezeichnet $\delta^{13}C_{DIC(GA)}$ das Kohlenstoffisotopenverhältnis des DIC im Grundwasseranstrom der Kontamination. $\delta^{13}C_{DIC(Q)}$ steht für das Kohlenstoffisotopenverhältnis des während der Schadstoffmineralisierung gebildeten DIC. Dabei wird angenommen, dass $\delta^{13}C_{DIC(Q)}$ die Isotopensignatur des nicht abgebauten

Schadstoffs (Ausgangssubstrats) in der Quelle trägt (Hunkeler et al., 1999b). $\delta^{13}C_{DIC(M)}$ entspricht dem $^{13}C/^{12}C$-Verhältnis des DIC, das innerhalb der Schadstofffahne gemessen wurde. A und (1-A) stellen die Mischungsanteile der beiden Ausgangsisotopenverhältnisse ($\delta^{13}C_{DIC(GA)}$ und $\delta^{13}C_{DIC(Q)}$) dar, aus denen $\delta^{13}C_{DIC(M)}$ resultiert. Anhand der DIC-Konzentration im Grundwasseranstrom der Schadstofffahne ($C_{DIC(GA)}$) und mit Hilfe von A kann auf die Menge des in der Schadstofffahne gebildeten DIC ($C_{DIC(B)}$) geschlossen werden (Gleichung 1-8).

Gleichung 1-8
$$C_{DIC(B)} = C_{DIC(GA)} \frac{(1-A)}{A}$$

Schließlich ist eine Abschätzung der mineralisierten Schadstofffraktion ($C_{Schadstoff}$) anhand des gebildeten DIC ($C_{DIC(B)}$) möglich (Gleichung 1-9).

Gleichung 1-9
$$C_{Schadstoff}\,[M] = \frac{C_{DIC(B)}}{Z_C}$$

Z_C bezeichnet die Anzahl der Kohlenstoffatome des Schadstoffmoleküls, die bei der Mineralisierung umgesetzt werden.

2.5 Ergebnisse

Die Messstelle Saf ZZ 23/00 im nordöstlichen Abstrom der Schadstofffahne (Fig. 2-1) wies in Monitoringkampagnen der letzten Jahre BTEX-Konzentrationen in einer Schwankungsbreite von ca. 40 bis 700 mg L^{-1} auf. Im Dezember 2004 wurde mit einem MLPS die vertikale hydrogeochemische Struktur der BTEX-Schadstofffahne untersucht und der *in situ* Benzolabbau anhand der Isotopensignatur des Benzols und DIC charakterisiert. Um den mikrobiellen Benzolabbau innerhalb der verschiedenen geochemischen Zonen untersuchen zu können, wurde parallel ein BACTRAP-Experiment in acht Tiefen durchgeführt.

2.5.1 Geochemie

Benzol stellt mit einem Anteil von mehr als 99 % die wesentliche BTEX Komponente dar. Toluol, Ethylbenzol sowie die Xylole überschreiten als Summenparameter in keinem Beprobungshorizont Gesamtkonzentrationen von 2 mg L^{-1} (nicht dargestellt). Die Benzolfahne ist vertikal strukturiert (Fig. 2-4). Der maximale Benzolgehalt von > 6.400 µmol L^{-1} (> 500 mg L^{-1}) wurde in 14 m Tiefe bestimmt. Zum oberen und unteren Schadstofffahnenrand nehmen die Benzolkonzentrationen bis auf 415 bzw. 1670 µmol L^{-1} kontinuierlich ab.

Die Isotopensignatur des Benzols weist in den mittleren Tiefen $\delta^{13}C$-Werte zwischen -28,0 ‰ und -29,0 ‰ auf (Fig. 2-4), die im wesentlichen den Werten des Benzols im Schadenszentrum (-28,5 bis -29,5 ‰) entsprechen (Fischer et al., 2004; Vieth et al., 2005). In dieser Zone der Kontaminationsfahne kann daher über die Isotopenfraktionierungsmethode kein bzw. nur ein geringer biologischer Abbau von Benzol nachgewiesen werden (Tab. 2-1). Im oberen und unteren Bereich des Vertikalprofils der Messstelle Saf ZZ 23/00 ist eine ^{13}C-Anreicherung in der residualen Benzolfraktion um 3 bzw. 6 ‰ festzustellen, die offensichtlich auf Abbauprozesse hinweist. Eine Quantifizierung des prozentualen biologischen Schadstoffabbaus (B %) wurde unter Annahme sulfatreduzierender bzw. methanogener Bedingungen durchgeführt. Die Berechnung basiert auf den von Mancini et al. (2003) für den Benzolabbau bestimmten Fraktionierungsfaktoren (α), mit α von 1,0036 für sulfatreduzierende und α von 1,0019 für methanogene Abbaubedingungen. Der mikrobielle Benzolabbau unter der Verwendung der schweren Isotopensignatur der Quelle von -28,5 und dem höheren Isotopenfraktionierungsfaktor für sulfatreduzierende Bedingungen (1,0036) zeigt Abbaugrade bis zu 59 % am oberen und bis 83 % am unteren Fahnenrand (Tab. 2-1). Die Berechnung mit dem Fraktionierungsfaktor für methanogene Bedingungen (1,0019) unter Annahme einer relativ leichten Benzolquelle (-29,5 ‰) ergibt einen entsprechend höheren Abbau von 89 bis 98 % für die Fahnenränder. Mit Hilfe der hier berechneten Variabilität der Abbaugrade und den gemessenen Benzolkonzentrationen (C_t) in den einzelnen Tiefen der Messstelle Saf ZZ 23/00 konnte auf die theoretischen Schadstoffkonzentrationen der Kontaminationsquelle (C_0) zu Beginn eines biologischen Abbaus (t=0) zurückgerechnet werden (Tab. 2-1). Die Berechnung des Benzolabbaus für sulfatreduzierende Bedingungen zeigte, dass diese theoretisch bestimmten Konzentrationen von > 10.000 µmol L^{-1} im unteren Aquiferbereich in etwa mit den Benzolkonzentrationen der Schadstoffquelle (>12.800 µmol L^{-1}) vergleichbar sind. Unter der Annahme methanogener Abbaubedingungen ergeben sich hypothetische Benzolkonzentrationen für die Schadstoffquelle von > 90.000 µmol, die die physikalische Löslichkeit des Benzols im Wasser überschreiten und daher unrealistisch sind. Das unterstreicht frühere Ergebnisse, nach denen die Methanogenese im Vergleich zur Sulfatreduktion am Standort keinen wesentlichen Anteil am Benzolabbau hat (Fischer et al., 2004). Unter sulfatreduzierenden Bedingungen ergibt sich ein Benzolabbau von bis zu 9.000 µmol L^{-1} (ca. 700 mg L^{-1}) auf dem Fließweg zwischen Quelle und Messpunkt im unteren Fahnenbereich, welcher aufgrund der hohen Benzolkonzentrationen im Schadenszentrum durchaus möglich sein könnte. Im oberen Rand der Fahne ergibt sich ein entsprechend geringerer Benzolabbau zwischen 200 bis 700 µmol L^{-1} (16-55 mg L^{-1}).

In unbelasteten Bereichen des Aquifers werden Sulfatkonzentrationen von > 10.000 µmol L^{-1} nachgewiesen, so dass Sulfat den wichtigsten Elektronenakzeptor für mikrobielle

Abbauprozesse darstellt (Fischer et al., 2004; Vieth et al., 2005). Im Vertikalprofil schwanken die Sulfatkonzentrationen zwischen ca. 20 µmol L^{-1} in einer Tiefe von 14 m und > 5.000 µmol L^{-1} am oberen Fahnenrand (Fig. 2-4). Verglichen mit unbelasteten Bereichen des Aquifers deutet die Abnahme des Sulfats infolge einer mikrobiellen Sulfatreduktion auf eine deutliche Sulfatsenke in der Fahne hin.

Tab. 2-1: Berechnung des biologischen Abbaus [B%] für sulfatreduzierende (αS = 1.0036) und methanogene Bedingungen (αM = 1.0019) unter Berücksichtigung der Schwankungsbreite der Isotopensignatur von Benzol in der Quelle (R_0 = -28,5 ‰ bis -29,5 ‰)

Tiefe [m]	C_{Benzol} [µmol L^{-1}]	$\delta^{13}C_{Benzol}$ [‰]	B %(S)[1] [%]	B %(M)[2] [%]	C_0 (S)[3] [µmol L^{-1}]	C_0(M)[4] [µmol L^{-1}]
10,4	415	-25,4 ± 0,4	59	89	1013	3857
11,4	2557	-28,2 ± 0,2	9	51	2795	5208
12,2	4901	-28,0 ± 0,4	13	55	5608	10886
13,2	6159	-28,6 ± 0,2	-4	37	5916	9825
14,2	6419	-27,4 ± 0,2	26	68	8725	19743
15,6	1840	-22,3 ± 0,2	83	98	10871	91177
16,7	1668	-22,3 ± 0,4	83	98	9916	83647

[1] Berechnung des biologischen Abbaus für sulfatreduzierende Bedingungen unter Annahme von R_0 = -28,5 ‰
[2] Berechnung des biologischen Abbaus für methanogene Bedingungen unter Annahme von R_0 = -29,5 ‰
[3] Berechnung der Benzolkonzentration der Quelle auf Grundlage von B %(S)[1]
[4] Berechnung der Benzolkonzentration der Quelle auf Grundlage von B %(M)[2]

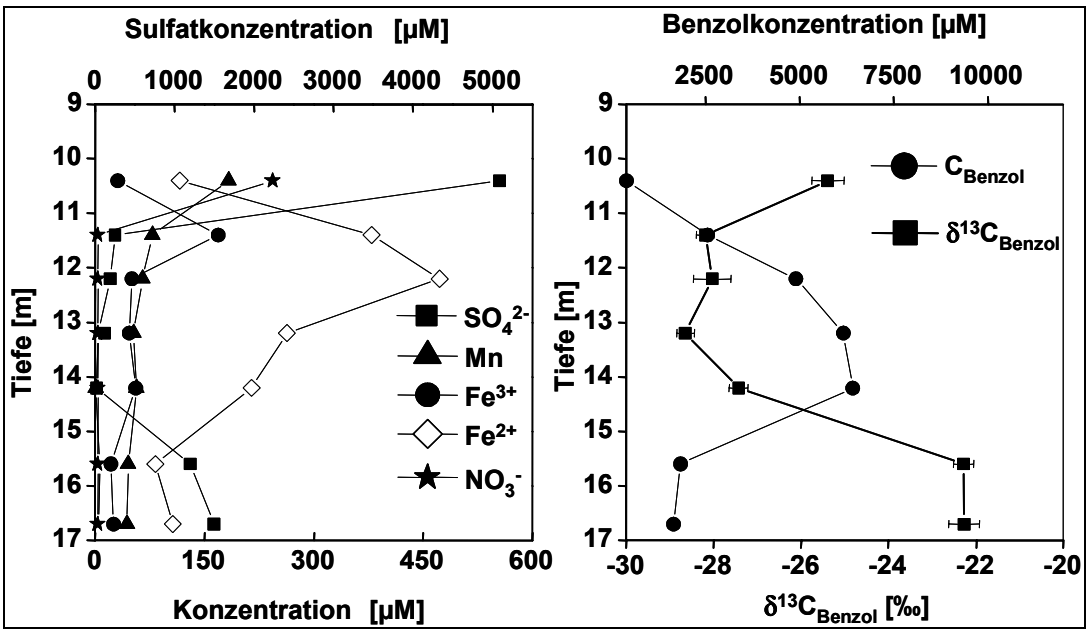

Fig. 2-4: Links vertikale Verteilung der Eisen(II)-(◊), Eisen(III)-(●), Nitrat-(*), Mangan-(▲), Sulfat-(■), und rechts der Benzolkonzentrationen (●) sowie der Kohlenstoffisotopenverhältnisse des Benzols (■).

Der höchste Nitratgehalt von 244 µmol L^{-1} wurde am oberen Rand der Fahne im Übergangsbereich zur ungesättigten Zone festgestellt (Fig. 2-4). In tieferen Zonen konnte

kein Nitrat nachgewiesen werden. Im Vergleich zum Sulfat steht für Abbauprozesse Nitrat als Elektronenakzeptor also nur in der oberen Zone des Aquifers in relativ geringen Konzentrationen zur Verfügung.

Die Konzentrationen von Mangan betragen etwa 180 µmol L^{-1} am oberen Rand der Fahne und reduzieren sich mit zunehmender Tiefe kontinuierlich auf Konzentrationen um 44 µmol L^{-1} (Fig. 2-4). Eine Manganreduktion könnte in der oberen Zone der Kontaminationsfahne einen Beitrag zum Schadstoffabbau liefern. Insgesamt sind die Mangankonzentrationen für einen signifikanten Schadstoffabbau jedoch zu gering.

Die Konzentrationen von Eisen(III) liegen im Bereich von ca. 20-60 µmol L^{-1} mit Ausnahme von ca. 170 µmol L^{-1} in einer Tiefe von 11,5 m. Die Eisen(II)-Werte schwanken zwischen etwa 80-470 µmol L^{-1} (Fig. 2-4). Erhöhte Fe(II)-Konzentrationen zwischen 11-14 m weisen auf eine Fe(II)-Mobilisierung hin, die durch Abbauprozesse verursacht sein könnte. Auffällig ist, dass die Bereiche erhöhter Fe(II)-Gehalte (11-14 m) durch die geringsten Sulfid-Konzentrationen gekennzeichnet sind. Diese liegen zwar generell bei ≤ 6 µmol L^{-1} (nicht dargestellt), aber die Fällung von Fe(II) durch Sulfid könnte zu einer Unterschätzung der Eisenreduktion beitragen. Gefälltes Eisensulfid verbleibt an der Aquifermatrix und kann im Grundwasser nicht quantitativ nachgewiesen werden, so dass der Anteil der Eisenreduktion anhand der Eisenmobilisierung kaum quantifizierbar ist. Es kann aber grundsätzlich davon ausgegangen werden, dass Eisen(III) am Standort keinen bedeutenden Elektronenakzeptor für Abbauprozesse darstellt (Wachter, 2004).

Die Sauerstoffkonzentrationen liegen in der Messstelle mit Ausnahme des Übergangsbereichs zur ungesättigten Zone unterhalb von 40 µmol L^{-1}, so dass Sauerstoff als Elekronenakzeptor in tieferen Zonen der Fahne nicht zur Verfügung steht (nicht dargestellt).

Die DIC-Konzentrationen verhalten sich proportional zum Schadstoff (Fig. 2-5). Die höchsten Konzentrationen von > 17.000 µmolC L^{-1} wurden für den am stärksten kontaminierten Bereich zwischen 11-14 m registriert und nehmen am oberen und unteren Fahnenrand ab. Die maximal gemessenen DIC-Werte des Vertikalprofils entsprechen in der Größenordnung den DIC-Konzentrationen, die für die Hauptschadenszonen der BTEX-Fahne ermittelt wurden (Fischer et al., 2004). Im unteren Bereich der Fahne geht die DIC-Konzentration bis auf rund 1.300 µmolC L^{-1} zurück. Die Verteilung der verschiedenen Spezies des DIC im Grundwasser ist pH-Wert abhängig. Im Bereich der Schadstofffahne wurden pH-Werte von 6,5 - 7,0 bestimmt, so dass entsprechend des Kalk-Kohlensäure-Gleichgewichts davon auszugehen ist, dass Hydrogencarbonat die dominierende Spezies des DIC am Standort darstellt und Fällungsreaktionen ausgeschlossen werden können (Clark and Fritz, 1997). Dieser Trend wird auch im Tiefenprofil der Messstelle Saf ZZ 23/00 beobachtet, wo neutrale pH-Werte von ca. 7,0 für alle Tiefen bestimmt wurden.

Unter der Annahme, dass die Sulfatreduktion der dominierende Prozess des mikrobiellen Schadstoffabbaus am Standort ist (Wachter, 2004), kann folgende Abschätzung vorgenommen werden. Nach Wiedemeier et al. (1999) werden bei der Mineralisierung von 1.000 µmol Benzol unter der Bildung von 6.000 µmol CO_2 3.750 µmol Sulfat verbraucht (Wiedemeier et al., 1999). Eine mikrobielle Oxidation von rund 1.300 µmol L^{-1} Benzol unter Bildung von etwa 8.000 µmolC L^{-1} DIC kann somit theoretisch eine Sulfatsenke von ca. 5.000 µmol L^{-1} bewirken, wenn der Aufbau zelleigener Biomasse unberücksichtigt bleibt. Verglichen mit der DIC-Konzentration von ca. 8.300 µmolC L^{-1} im Anstrom der BTEX-Fahne konnte eine maximale Konzentrationszunahme um ca. 10.000 µmolC L^{-1} im stark kontaminierten Bereich der Messstelle Saf ZZ 23/00 beobachtet werden. Aus der maximalen Differenz der DIC-Konzentrationen im Grundwasseranstrom und im Bereich der Messstelle Saf ZZ 23/00 berechnet sich ein theoretisches Abbaupotenzial von ca. 1.700 µmol L^{-1} Benzol. Aus den beiden Berechnungsansätzen resultiert ein Unterschied von etwa 400 µmol Benzol.

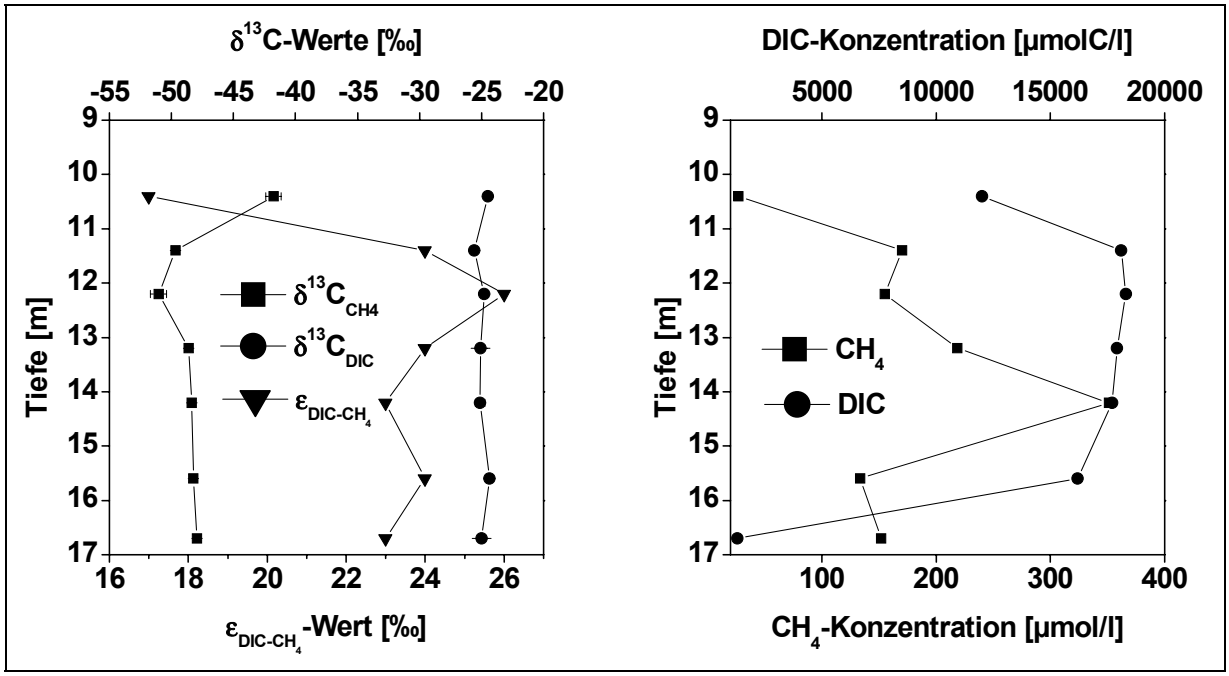

Fig. 2-5: Vertikale Verteilung der Kohlenstoffisotopenverhältnisse von Methan [■], DIC [●] und den Anreicherungsfaktoren [▼] (links) sowie der Methan- [■] und DIC-Konzentrationen [●] (rechts) in der Messstelle Saf ZZ 23/00.

In kontaminierten Aquiferen gilt die Zunahme von Methan als ein Indiz für Biodegradation unter methanogenen Milieubedingungen (Wiedemeier et al., 1999). Die CH_4-Konzentrationen im Grundwasser können außerdem durch Methanoxidation beeinflusst werden (Breukelen and Griffioen, 2004; Grossmann et al., 2002). Die Methanogenese führt zur Bildung von isotopisch leichtem Methan während die aerobe Methanoxidation mit einer Anreicherung schwerer Isotope verbunden ist. Um Methanbildungs- oder Abbauprozesse voneinander zu unterscheiden, kann der Anreicherungsfaktor $\varepsilon_{DIC\text{-}CH4}$, die Differenz zwischen der Kohlenstoffisotopensignatur des DIC und Methans, genutzt werden (Fischer et al., 2004).

Geringe $\varepsilon_{DIC\text{-}CH4}$-Werte von < 20 ‰ gelten als Indiz für Methanoxidation, höhere Anreicherungsfaktoren deuten auf Methanbildung hin (Acetogenese 10-50 ‰; > 50 ‰ CO_2-Reduktion) (Fischer et al., 2004; Whiticar, 1999). Die berechneten Anreicherungsfaktoren für das Tiefenprofil der Messstelle Saf ZZ 23/00 variieren von 17-26 ‰ (Fig. 2-5). Im Beprobungshorizont bei 10,4 m wurden sowohl die geringsten Methankonzentrationen als auch die geringsten Anreicherungsfaktoren bestimmt, so dass im Übergang zur ungesättigten Zone des Aquifers Methanoxidation denkbar wäre. Mit zunehmender Tiefe trägt wahrscheinlich die Methanogenese zum Abbau der BTEX bei und führt zu einer Erhöhung der CH_4-Konzentration auf ca. 350 µmol L^{-1}, was etwa einem Abbau von 93 µmol (7 mg L^{-1}) Benzol entspricht (Wiedemeier et al., 1999). Wie anhand der Anreicherungsfaktoren abgeleitet werden kann, ist die Acetatgärung hierbei vermutlich der dominierende Methanbildungsprozess. Die relativ niedrigen Methankonzentrationen insgesamt zeigen jedoch, dass die Methanogenese keinen signifikanten Beitrag am Schadstoffabbau liefert.

Bei der Mineralisation von Benzol entsteht Kohlendioxid, das die gleiche Isotopensignatur wie das Ausgangssubstrat trägt (Fischer et al., 2004; Hunkeler et al., 2001). Im Anstrom der Fahne liegt die natürliche DIC-Isotopensignatur im Mittel bei -19,5 ‰ (Fischer et al., 2004), im Bereich der Messstelle Saf ZZ 23/00 sind die $\delta^{13}C_{DIC}$-Werte hingegen durchschnittlich um ca. 5,5 ‰ leichter (-24,5 bis -25,6 ‰) und zeigen keine markanten Unterschiede über die Tiefe (Fig. 2-5). Werte dieser Größenordnung sind generell im stärker kontaminierten Bereich der BTEX-Fahne beobachtet worden (Fischer et al., 2004). Anhand dieser Veränderung des Isotopensignals über die Fließstrecke wurde die Umsetzung von mineralisiertem Benzol berechnet (Gleichung 1-7 - Gleichung 1-9). Als Kohlenstoffisotopenverhältnis ($\delta^{13}C_{DIC(GA)}$) bzw. Konzentration ($C_{DIC(GA)}$) des DIC im Grundwasseranstrom wurde ein mittlerer Wert von -19,5 ‰ für $\delta^{13}C_{DIC(GA)}$ bzw. 8.300 µmolC L^{-1} für $C_{DIC(GA)}$ angenommen (Fischer et al., 2004). Das durchschnittliche Isotopenverhältnis des DIC im Profil der Messstelle Saf ZZ 23/00 ($\delta^{13}C_{DIC(M)}$) betrug ca. -25,0 ‰. Unter der Annahme identischer Isotopensignaturen des Benzols im Schadenszentrum und des DIC, das infolge einer Mineralisierung des Schadstoffs entsteht, wurde für die Quantifizierung ein $\delta^{13}C_{DIC(Q)}$ Wert von -28,5 ‰ angenommen, da im Fall einer vollständigen Mineralisierung die Isotopenbilanz geschlossen ist. Die Abschätzung ergibt für die Fließstrecke zwischen dem Zentrum der Schadstofffahne und der Messstelle Saf ZZ 23/00 eine Mineralisierung von etwa 2.160 µmol L^{-1} Benzol (169 mg L^{-1}).

Beim aeroben Benzolabbau kann eine Isotopenfraktionierung zwischen nicht abgebautem Benzol und gebildetem DIC von bis zu 4 - 6 ‰ auftreten (Hunkeler et al., 2001), wenn Biomasse gebildet wird oder der Abbau unvollständig ist. Die Fraktionierung zwischen Benzol und DIC beim anaeroben Abbau ist noch nicht untersucht. Würde eine Fraktionierung

zwischen nicht abgebauten Benzol und gebildetem DIC von maximal 6 ‰ ($\delta^{13}C_{DIC(Q)}$ = -34,5 ‰) bei der Quantifizierung des anaeroben Benzolabbaus berücksichtigt werden, so verringert sich die Menge des mineralisierten Benzols (808 µmol L^{-1} bzw. 63 mg L^{-1}) erheblich. Die Abschätzung des Abbaus setzt voraus, dass die Quellen organischen Materials für das DIC isotopisch charakterisiert sind. Am Standort Zeitz steht vorherrschend Benzol zur Verfügung. Wenn andere Quellen wie natürlicher gelöster Kohlenstoff (DOC) oder andere Schadstoffe zur DIC-Bildung beitragen, ist eine Abschätzung schwierig. Es ist daher offensichtlich, dass die Abschätzung des Abbaupotenzials über eine Isotopenbilanz mit Unsicherheiten verbunden ist.

2.5.2 Ergebnisse der tiefenorientierten Untersuchungen mit den BACTRAPs

Die BACTRAPs mit und ohne Benzolbeaufschlagung (^{12}C-Benzol, ^{13}C-Benzol, Kontrolle) zeigen für alle Beprobungshorizonte vergleichbare Fettsäuremuster. Dominierende Fettsäuren sind C16:0 (> 40 %) und C18:0 (> 20 %) sowie die ungesättigten Fettsäuren C16:1 und C18:1 mit einem prozentualen Anteil von jeweils ca. 10 %. Untergeordnet, in geringen Konzentrationen (< 10 %), konnten die Fettsäuren C14:0, C15:0, i-C15:0, a-C15:0, C18:2 sowie eine weitere C18:1 Fettsäure mittels GC-MS identifiziert werden.

Da sich die Fettsäuremuster der mit Benzol beladenen und unbeladenen BACTRAPs nicht wesentlich voneinander unterscheiden, konnte weder ein Substrateinfluss noch eine Abhängigkeit des mikrobiellen Konsortiums vom geochemischen Milieu in den einzelnen Tiefen festgestellt werden. Die Fettsäuren liefern daher keinen Hinweis auf eine unterschiedliche Besiedlung der BACTRAPs in den verschiedenen Beprobungshorizonten.

Verglichen mit einem BACTRAP-Experiment, das in einer Messstelle im weiter entfernten Grundwasserabstrom durchgeführt wurde (Geyer et al., 2005), sind die Fettsäuremuster im Bereich der Messstelle Saf ZZ 23/00 homogener und weniger komplex. Das lässt auf eine geringere Biodiversität auf dem BACTRAP schließen.

Die Kohlenstoffisotopensignaturen der Fettsäuren ($\delta^{13}C_{FA}$) aus dem Kontrollexperiment variieren zwischen -23 und -34 ‰. Die Isotopenanalysen der Proben aus dem Markierungsexperiment zeigten die höchsten ^{13}C-Anreicherungen (zwischen 28 und 468 ‰) für die C16:1 Fettsäure (Fig. 2-6). Eine etwas geringere Markierung wurde für die Fettsäuren i-C15:0, C14:0, C16:0 und C18:1 (-20 ‰ bis 145 ‰) bestimmt. Die signifikante ^{13}C-Anreicherung von Fettsäuren ist auf die Transformation von ^{13}C aus dem Benzol in die Biomasse zurückzuführen. Keine signifikante Transformation der ^{13}C-Markierung des Benzols wurde in die a-C15:0, C15:0 und C18:0 mit $\delta^{13}C_{FA}$-Werten von -14 bis -25 ‰ beobachtet (Fig. 2-6).

Die Isotopensignatur der Fettsäuren einer Spezies spiegelt, abgesehen von geringen Unterschieden, die auf Isotopenfraktionierungsprozesse beruhen, im Wesentlichen das

Isotopenverhältnis des Wachstumssubstrates wider (Abraham et al., 1998). Auch in Experimenten mit markierten Kohlenstoffsubstraten ist die Isotopensignatur bei PLFA verschiedener Kettenlänge etwa gleich (Pelz et al., 2001a) und auch kaum vom Wachstumsstadium beeinflusst (Abraham et al., 1998). Der Kohlenstoff wird also etwa gleichermaßen zum Aufbau von Membranlipiden verwendet. Die unterschiedlich starke Markierung zeigt daher die Besiedlung des BACTRAPs durch mikrobielle Konsortien, welche ^{13}C-Benzol unterschiedlich effektiv als Kohlenstoffquelle nutzen. Zum Beispiel scheinen Organismen, die unmarkierte C18:0, a-C15:0 oder C15:0 Fettsäuren besitzen, das markierte Benzol nicht oder nur kaum zu metabolisieren und bevorzugen andere Kohlenstoffquellen zur Synthese. Als zusätzliche Kohlenstoffquellen, die den Mikroorganismen zur Verfügung stehen können und die durch eine natürliche Isotopensignatur gekennzeichnet sind, kommen aromatische Kohlenwasserstoffe wie zum Beispiel BTEX aus der Kontamination sowie auch DOC in Frage.

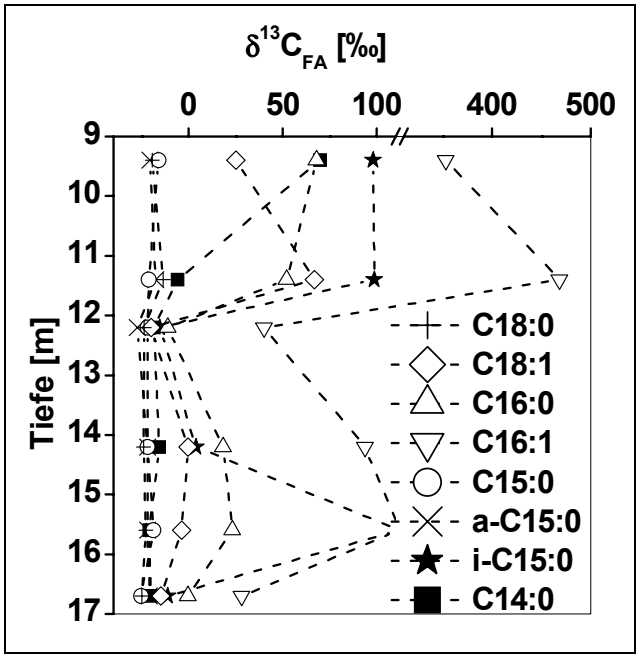

Fig. 2-6: Übersicht der Isotopensignaturen der Fettsäuren $\delta^{13}C_{FA}$ [‰] in den einzelnen Beprobungshorizonten. Die Abkürzungen und Nomenklatur der Fettsäuren sind im Kapitel Material und Methoden (2.4.9) erklärt.

Anhand der Intensität der ^{13}C-Anreicherung in den Fettsäuren konnten Zonen unterschiedlicher mikrobiologischer Aktivität identifiziert werden. Im obersten Bereich der untersuchten Messstelle (1. und 3. Tiefe) zeigt die isotopische Anreicherung in der C16:1 von >350 ‰ demnach die größte mikrobielle Aktivität an. Im mittleren Tiefenintervall (12 m) wurden die geringsten Markierungen (40 ‰) für C16:1 nachgewiesen. Im unteren Bereich der Messstelle (14-15 m) zeigt die C16:1 mittlere ^{13}C-Anreicherung von ca. 94 ‰ (14,2 m) bzw. 188 ‰ (15,6 m). Die Ergebnisse verdeutlichen, dass nicht nur in den Randbereichen

der Schadstofffahne sondern auch in den stark benzolbelasteten Zonen des Aquifers ein mikrobieller Benzolabbau stattfindet.

2.6 Diskussion

2.6.1 Geochemie

Die Abschätzung des Benzolabbaus anhand der Isotopensignaturen des Benzols bzw. DIC und Methan hat gezeigt, dass bevorzugt im oberen und hauptsächlich im unteren Randbereich der Fahne ein *in situ* Abbau stattfindet. Im Tiefenintervall von ca. 12-14 m geben die $\delta^{13}C_{Benzol}$-Werte keinen Hinweis auf mikrobiellen Abbau, da sie ungefähr das Isotopensignal des Quellbereichs (-28,5‰) tragen. Vermutlich ist im Bereich der sehr hohen BTEX-Konzentrationen der Anteil des mikrobiell abgebauten Benzols im Verhältnis zum nicht umgesetzten zu gering, um eine Veränderung der Isotopensignatur eindeutig nachweisen zu können.

Die konservative Abschätzung des Abbaus auf dem Fließweg zwischen Quelle und Messpunkt anhand der Isotopenmethode ergab für sulfatreduzierende Bedingungen einen Benzolabbau von bis zu 9.000 µmol L^{-1} (ca. 700 mg L^{-1}). Der gemittelte Abbau über alle Tiefen liegt bei ca. 3.000 µmol L^{-1} (ca. 230 mg L^{-1}). Mit Hilfe der Konzentrationen und Isotopensignaturen des DIC wurde ein Benzolabbau von etwa 2.160 µmol L^{-1} (ca. 169 mg L^{-1}) abgeschätzt. Aus der Elektronenbilanz über die Sulfatsenke ergab sich ein Abbaupotenzial von 1.700 µmol L^{-1} (ca. 133 mg L^{-1}) Benzol. Etwa 350 µmol L^{-1} (ca. 28 mg L^{-1}) Benzol könnten zusätzlich über methanogene Prozesse abgebaut worden sein. In der Summe errechnet sich ein möglicher Benzolabbau infolge Sulfatreduktion und Methanogenese von 2.050 µmol (ca. 160 mg). Dieser Wert entspricht etwa der Abschätzung der Mineralisation über das DIC. Die Quantifizierung des Abbaus anhand der Benzolisotope liegt etwa um ein Drittel höher aber in der gleichen Größenordnung. Die verschiedenen Berechnungsansätze liefern in der Größenordnung übereinstimmende Ergebnisse, so dass die Werte für den berechneten Benzolabbau realistisch erscheinen.

Eine Diskussion der Abweichungen der verschiedenen vorgestellten Ansätze zur Abschätzung des anaeroben Benzolabbaus gestaltet sich schwierig. Das Karbonatsystem (DIC) könnte durch Fällungen beeinflusst werden. Dies scheint aufgrund neutraler pH-Werte am Standort jedoch unwahrscheinlich zu sein. Ausgasungen in erheblichem Umfang sind nur im Bereich der Zonen mit direktem Kontakt zur ungesättigten Bodenzone zu erwarten und über die Mächtigkeit des gesamten Aquifers eher als gering anzusehen. In der Elektronenbilanz fehlt im Wesentlichen die Eisenmobilisierung, weil Fe(II) unter sulfatreduzierenden Bedingungen gefällt wird und für quantitative Abschätzungen nicht erfasst werden kann.

Die Beurteilung des mikrobiellen Schadstoffabbaus über die Methode der Isotopenfraktionierung gibt vermutlich eine realistische Größe für den Abbau zwischen Quelle und Messpunkt. Die Methode führt eher zu einer konservativen Abschätzung, wobei der mikrobielle Abbau nicht wesentlich überschätzt werden sollte. Mischungsprozesse auf dem Fließweg von Grundwasserströmen mit einerseits kaum und andererseits stark abgebauten Schadstoffmengen können zu einer Unterschätzung des tatsächlichen Abbaus führen (Fischer et al., 2004; Richnow et al., 2003a). Zu einer Überschätzung des Abbaus führt die Isotopenmethode in der Regel nicht.

2.6.2 BACTRAP

Die Auswertung des BACTRAP-Experiments hat gezeigt, dass in allen untersuchten Tiefen ein Einbau von ^{13}C-Kohlenstoff in die Fettsäuren erfolgte. Es konnte daher nachgewiesen werden, dass über den gesamten Bereich des untersuchten Vertikalprofils Benzolabbau stattfindet. Wie aufgrund der unterschiedlichen Intensität der Markierung abgeleitet werden kann, scheint der mikrobielle Benzolabbau offensichtlich in den Bereichen der maximalen Schadstoffbelastungen geringer als in den Randbereichen der Fahne zu sein. Da mit den BACTRAPs der mikrobielle Benzolabbau auch in stark kontaminierten Zonen nachgewiesen werden konnte, ist die Sensitivität dieses Testsystems wesentlich höher als beim Nachweis der Biodegradation über die Methode der Isotopenfraktionierung.

Die Quantifizierung des Benzolabbaus und Erstellung von Stoffumsatzbilanzen mit der Methode der BACTRAPs gestaltet sich problematisch. BACTRAPs verlieren unter vergleichbaren Bedingungen zu diesem Experiment etwa 80 bis 85 % der Menge des beaufschlagten Substrats (Benzol bzw. Toluol) (Geyer et al., 2005). Ein Teil des Substrats wird im Wasser gelöst und mit dem Grundwasserstrom abtransportiert. Dieser Verlust ist nur sehr schwer abschätzbar. Qualitativ konnte in anderen Multilevel-Versuchen eine Desorption des ^{13}C-markierten Substrats aus den BACTRAPs nachgewiesen werden. BACTRAPs mit nicht markierten Substraten, die in ca. 25 cm Entfernung zu ^{13}C-markierten BACTRAPs exponiert waren bzw. Grundwasserproben aus ähnlicher Tiefe zeigten eine ^{13}C-Anreicherung (unveröffentlichte Ergebnisse). Zukünftig sind Referenzversuche mit inerten, nicht abbaubaren Substraten zur Bestimmung der Desorptionskinetik, zur Berechnung des den Mikroorganismen tatsächlichen zur Verfügung stehenden markierten Substrats sowie zur Abschätzung von Verlusten nötig.

Ein weiterer Teil des Substrats wird offensichtlich mineralisiert und als CO_2 bzw. Methan aus dem BACTRAP in den Grundwasserstrom abgegeben und steht somit zur Quantifizierung einer geschlossenen Massenbilanz nicht zur Verfügung. Da Mikroorganismen in der Regel einen bestimmten Teil des Substrates, auf dem sie wachsen, zur Synthese eigener Biomasse verwenden, könnten zur Abschätzung der Schadstofftransformation

Ertragskoeffizienten dienen. Ertragskoeffizienten beschreiben das Verhältnis zwischen Mineralisierung und Bildung von Biomasse und könnten in geschlossenen Modellsystemen bestimmt werden. *Ex situ* Referenzversuche im Labor sind allerdings schwierig, da anaerob Benzol abbauende Mikroorganismen gegenwärtig nur schwer kultivierbar sind. Alternativ könnte eine Abschätzung der Ertragskoeffizienten für die verschiedenen terminalen Elektronenakzeptoren mittels Referenzkulturen in Versuchen mit leichter abbaubaren Substraten wie Toluol oder Acetat bestimmt werden, die dann zunächst als Richtwerte für verschiedene biogeochemische Bedingungen zur Verfügung stehen.
Zukünftig soll anhand der Isotopensignatur der Anteil des in die Biomasse transformierten Substrats quantifiziert werden, so dass eine Abschätzung der Mineralisierung perspektivisch möglich scheint. Methoden zur Extraktion der gesamten Biomasse aus den *in situ* Mikrokosmen werden gegenwärtig entwickelt.
BACTRAPs stellen ein bedeutendes Testsystem zum direkten Nachweis der im Grundwasserleiter stattfindenden Biodegradation dar. Der entscheidende Vorteil des Testsystems ist, dass der Nachweis des mikrobiellen Schadstoffabbaus unter realen Aquiferbedingungen innerhalb nur weniger Monate erfolgen kann. Ein Nachteil zu Laborversuchen besteht derzeit in der eingeschränkten Möglichkeit zur Bilanzierung des Abbaus. Laborsysteme können geschlossene Stoffbilanzen einfach erfassen. Das ist, wie bereits dargestellt, mit *in situ* Mikrokosmen zurzeit nicht möglich. Da Testverfahren für Sedimente und Böden im Labor in der Regel nicht die Komplexität der Umweltbedingungen reproduzieren können, ist eine Übertragung der quantitativen Laborergebnisse auf den Aquifer ein kontrovers diskutiertes, ungelöstes Problem. Außerdem erfolgt der anaerobe Abbau meist sehr langsam, weshalb Laborverfahren häufig relativ aufwendig und zeitintensiv sind. Deshalb besitzen im Vergleich zu diesen herkömmlichen Systemen sowohl die Untersuchung der Isotopenfraktionierung als auch *in situ* Mikrokosmen mit isotopisch markierten Substanzen ein erhebliches Potenzial zur Charakterisierung des mikrobiellen *in situ* Schadstoffabbaus in kontaminierten Grundwasserleitern.

2.7 Zusammenfassung

Eine tiefenorientierte Untersuchung von Kontaminationsfahnen erlaubt die Analyse hydrogeochemischer Gradienten im Aquifer. Konzentrationen von Elektronenakzeptoren geben Aufschluss über vorherrschende biogeochemische Prozesse. Die vertikale Analyse der Kontaminationsfahne anhand der Konzentration und Isotopensignatur von Kontaminanten liefert qualitative und quantitative Indikatoren für den *in situ* Schadstoffabbau. Anhand der Methode der Isotopenfraktionierung konnten Abbauprozesse an den vertikalen Fahnenrändern des untersuchten Tiefenprofils analysiert werden. Eine messbare Veränderung der Isotopensignatur kann meist erst nach einem relativ hohen

Schadstoffumsatz nachgewiesen werden. Bei geringem Abbau ist die Isotopenmethode relativ unempfindlich. Zusätzliche Untersuchungen mit den BACTRAPs konnten hier weiterhelfen, da mit dieser Methode der Schadstoffabbau mit einer höheren Empfindlichkeit nachgewiesen werden konnte. Mit der Isotopenmethode ist eine quantitative Abschätzung des Abbaus auf dem Fließweg zwischen Quelle und Messpunkt möglich. Der Einsatz von *in situ* Mikrokosmen mit isotopisch markierten Substanzen kann als ein empfindliches Testsystem für den eindeutigen Nachweis des Abbaus genutzt werden. Es werden qualitative Informationen zum Abbaupotenzial in Bereich der untersuchten Messstelle gewonnen. Verglichen mit Abbauexperimenten im Labor geben *in situ* Mikrokosmen direkte Aussagen zur Biodegradation im Grundwasserleiter. Schwierigkeiten bestehen derzeit noch in der quantitativen Interpretation der Daten. Die vertikale Analyse von geochemischen Gradienten und Isotopensignaturen von Schadstoffen, DIC sowie Methan verbunden mit dem Einsatz von *in situ* Mikrokosmen liefert Informationen zu biogeochemischen Abbauprozessen, die für einen Schadensfall relevant sind. Der Einsatz von MLPS in Verbindung mit modernen geochemischen und isotopenchemischen Verfahren ermöglicht somit mit vertretbarem Aufwand die Gewinnung entscheidender Informationen für die Planung von *Natural Attenuation*-Vorhaben bzw. für die Überwachung von aktiven Sanierungs- bzw. *Enhanced Natural Attenuation*-Verfahren.

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3 *In situ* microcosms to evaluate natural attenuation potentials in contaminated aquifers

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3.1 Abstract

In situ microcosm (BACTRAP®) experiments with ^{13}C-labelled toluene and benzene were conducted to investigate the *in situ* biodegradation in contaminated aquifers at field sites with different geological and geochemical conditions. The transformation of the carbon (^{13}C), derived from labelled substrates, into fatty acids verified the assimilation of the pollutants with formation of biomass. This clearly demonstrated that the *in situ* microcosm system is a useful culture-independent method to investigate *in situ* biodegradation in the aquifer. In addition, metabolites such as benzylsuccinic acid were found on toluene amended BACTRAPs indicating that toluene was degraded anaerobically. This result corresponded to the geochemical conditions found at the field site and the approach enabled to analyse the metabolic pathway governing *in situ* toluene biodegradation in the polluted aquifer.

Phospholipid fatty acids (PLFA) of living cells make up a significant part of the total lipid fatty acid (TLFA) fraction. Comparing BACTRAPs exposed at different geochemical zones of the aquifers, the fatty acid composition was found to be relatively similar indicating that the composition of the TLFA was of low taxonomic value and not sensitive enough for a community analysis. Therefore the composition of the microbial communities were analysed by genetic profiling and sequencing of partial 16S rRNA genes PCR-amplified from total DNA, extracted directly from the BACTRAPs. Sequences retrieved from the BACTRAPs indicated a dominance of not-yet cultivated bacteria, with several of them phylogenetically closely related to those with an iron and sulphate reducing capacity, typically found at BTEX and mineral oil polluted sites.

3.2 Introduction

The fate of pollutants in contaminated aquifers is mainly governed by microbial processes. Abiotic processes such as dispersion, dilution and volatilisation may contribute to a decrease in concentration but do not lead to a significant reduction of the overall amount of contaminants. Therefore, the evaluation of *in situ* biodegradation is crucial for the implementation of Natural Attenuation (NA) concepts in groundwater management strategies. Conventionally, complex calculations of decreasing contaminant concentrations related to the depletion of electron acceptors have been used to identify biodegradation

processes. However, this method is difficult to apply in order to evaluate the fate of a single pollutant in contaminant mixtures on the field scale, particularly in the very heterogeneous natural environment with several competing electron acceptor-donor interactions. Except for the stable isotope fractionation technique (Meckenstock et al., 2004a) and relatively labour intensive tracer experiments (Fischer et al., 2005; Reusser and Field, 2002) tools to quantify the actual *in situ* biodegradation of organic contaminants in the aquifer are scarce.

Culture-dependent laboratory microcosm studies are often used to investigate the potential of *in situ* biodegradation. However, this approach is not reliable because the majority of microorganisms have not been cultured yet and even the reproducible cultivation of anaerobic bacteria degrading typical contaminants such as BTEX and PAH is not an easy task. Laboratory microcosm studies as well as percolation column experiments, simulating the contaminant degradation under controlled water flow conditions, are time consuming and the conditions in the laboratory are certainly different to the actual natural environment complicating the interpretation of the results. Several limitations are related to these laboratory approaches particularly in providing reliable data to transfer the results to the actual ongoing *in situ* processes.

Recently, ^{13}C-labelled substrates in combination with ^{13}C-enrichment of PLFA were used to characterise bacterial toluene degradation in soil, sediment or aquifer microcosms (Hanson et al., 1999; Pelz et al., 2001a; Pelz et al., 2001b) and to trace the assimilation of toluene along a food chain (Mauclaire et al., 2003) providing useful information on carbon fluxes and metabolites. At the field scale, microbial incorporation of ^{13}C-labelled acetate into biomarker molecules like PLFA and DNA were successfully used to detect microbes which were suggested to be responsible for the reduction of uranium(VI) (Chang et al., 2005). To monitor the *in situ* dynamics of microbial communities in a BTEX polluted aquifer a mesocosm study was designed (Hendrickx et al., 2005). In this study, uncontaminated aquifer material was incubated either in the uncontaminated area or nearby a contaminated area of the aquifer and it was shown that the bacterial communities were not identical in these two zones using molecular biological techniques.

To overcome the limitations of *ex situ* laboratory test systems and to improve the monitoring of *in situ* biodegradation, an *in situ* microcosm system (BACTRAP®) was developed that can be directly incubated within the groundwater monitoring wells (Geyer et al., 2005). The system consists of pellets containing activated carbon that can be loaded with ^{13}C-labelled contaminants. The pellets provide large amounts of interfacial area for colonisation or attached growth of degrading bacteria. If the indigenous bacteria colonise the BACTRAPs and consume the contaminants *in situ*, the ^{13}C-labelled carbon will be transformed into the biomass and can be traced within biomarker molecules such as fatty acids or nucleic acids.

In a first approach, BACTRAPs containing activated carbon beads were loaded with ^{13}C-labelled benzene and toluene prior to incubation within the anaerobic zone of a contaminated aquifer (Geyer et al., 2005; Stelzer et al., 2006b). The pattern of fatty acids (FA) and the incorporation of ^{13}C into FA provided evidence for the *in situ* degradation of the test substrates and the metabolic transformation within a complex microbial community. In order to enable a more detailed characterisation of the bacterial community involved in biodegradation, we now analysed the microbial community colonising the *in situ* microcosms. For that reason the total DNA was directly extracted from the *in situ* microcosms and molecular tools were applied to study the microbial community structure. Partial sequences of the bacterial 16S rRNA genes, covering approximately 30 % (400 base pairs) of the complete gene, were PCR-amplified from total DNA and the diversity of the amplified product was visualised by genetic profiling, using the single-strand conformation polymorphism technique (SSCP) (Dohrmann and Tebbe, 2004; Schwieger and Tebbe, 1998).

Because the adsorbing material of the BACTRAPs can enrich degradation metabolites we intended to identify the metabolic process and pathway governing the *in situ* biodegradation of toluene in the aquifer.

In this paper we present our recent progress with BACTRAP systems. We compare *in situ* microcosm experiments with different contaminants at different field sites. This *in situ* approach was used to monitor the biodegradation potential within different geochemical zones of contaminated aquifers related to the vertical structure of a contamination plume. In addition, perspectives to use *in situ* microcosm experiments for microbial community structure analysis and for the identification of metabolic pathways are discussed.

3.3 Materials and Methods

3.3.1 Chemicals

The chemicals and solvents were obtained in p.A. quality from Merck unless stated. [$^{13}C_6$] benzene, [^{13}C]-α-toluene and benzylsuccinate were obtained from Sigma-Aldrich (St. Louis, USA). [$^{13}C_7$] toluene was purchased from Chemotrade Leipzig (Germany). All the isotopically labelled compounds had a chemical and an isotope purity higher than 99 %.

3.3.2 Field Sites

Zeitz (Saxony-Anhalt, Germany). The benzene, toluene, ethylbenzene and *o,m,p*- xylene (BTEX) contaminated aquifer is located in the area of a former hydrogenation plant close to the city of Zeitz, Germany (Fig. 3-1). A detailed description of the site hydrogeology and hydrochemical conditions of the contamination plume is given in previous studies (Fischer et al., 2004; Vieth et al., 2005).

The BACTRAP experiments were performed in a multilevel approach in the upper aquifer to investigate different geochemical zones of the vertical BTEX plume. The thickness of the upper aquifer varies between 4 to 6 m. In the source area total BTEX concentrations exceeded 900 mg L^{-1} (Fig. 3-1). Benzene and toluene were present in concentrations up to 850 mg L^{-1} and 50 mg L^{-1}, respectively. Ethylbenzene and xylenes were typically present in concentrations lower than 3 mg L^{-1}.

At the test site the predominant electron acceptor used for biodegradation was sulphate (Vieth et al., 2005). Active sulphate reduction in the course of the plume was evident by an enrichment of ^{34}S-sulphate (Fischer et al., 2004). In addition, methanogenesis had a minor impact on microbial BTEX-transformation in the BTEX-plume but a more significant impact in the source area (Fischer et al., 2005). Other electron acceptors like oxygen, nitrate and iron play a minor role for the overall biodegradation processes at the site (Dethlefsen et al., 2004; Fischer et al., 2004; Vieth et al., 2005).

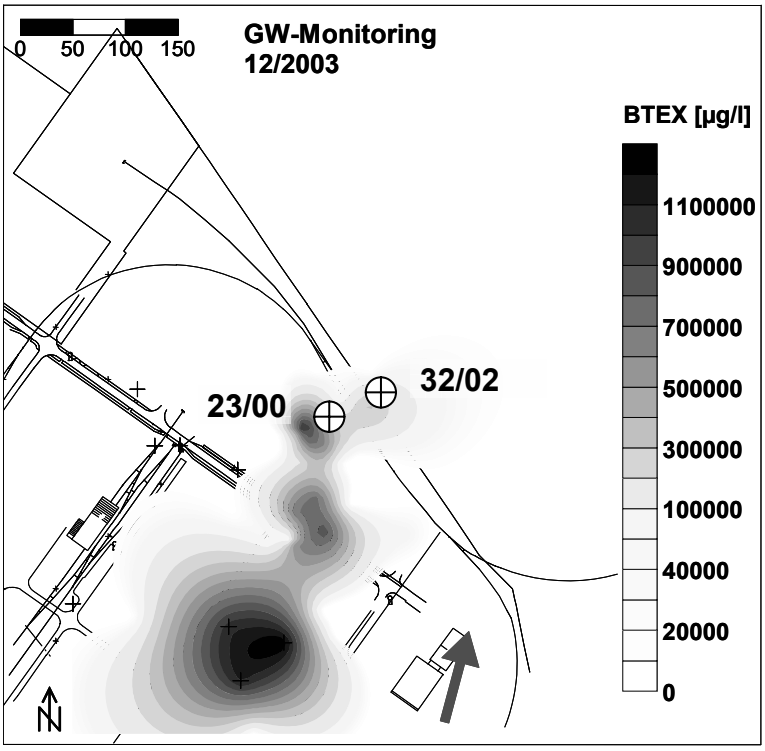

Fig. 3-1: Concentration of BTEX [µg L^{-1}] in the upper aquifer in the area of the former hydrogenation plant in Zeitz (Saxony-Anhalt, Germany). The locations of the *in situ* microcosm experiments are indicated. At well 23/00 the multilevel experiment was carried out and well 32/00 was used for the *in situ* microcosm study by Geyer et al. (2005). The arrow depicts the general groundwater flow direction.

Gneisenau (Dortmund, Germany). The anaerobic tar oil and BTEX contaminated aquifer is located at the former coking plant Gneisenau in the vicinity of a coal mine in the Ruhr area. The hydrological situation is characterised by two aquifers separated by erratic marls and tills. The hydraulic conductivity of the upper porous quaternary aquifer is relatively low at

the basis (k_f of about 10^{-9} m s^{-1}) but is significantly higher in sandy layers ($k_f = 10^{-6}$ to 10^{-4} m s^{-1}). The water table is located 5 to 20 m below ground. The lower aquifer is a confined fractured cretaceous aquifer. Both aquifers are contaminated by BTEX, PAH and other contaminants typical for coking plants. The upper quaternary aquifer was mainly contaminated by BTEX (up to 1.5 mg L^{-1}) and naphthalene (up to 750 µg L^{-1}), whereas in the deeper cretaceous aquifer mainly benzene (up to 45 mg L^{-1}) was found. Oxygen concentrations were always below 1 mg L^{-1}. The isotope enrichment of ^{34}S-sulphate in most contaminated areas provided evidence for sulphate reduction (Pfeifer, 2004). Sulphate, with concentrations up to 1660 mg L^{-1}, is likely the most important terminal electron acceptor at this site. Geochemical investigations suggested that methanogenesis as well as nitrate, iron and manganese reduction are probably less important for microbial processes in the subsurface (Pfeifer et al., 2003). The presence of benzylsuccinic acid, a typical metabolite of toluene, provided further evidence that anaerobic degradation processes are predominant at the site (Pfeifer, 2004).

3.3.3 Multi level packer systems used for depth discrete experiments

A multi level packer system (MLPS) was used for depth discrete sampling in Zeitz. The multi level packer system is a water-filled sock tube inserted into the well, which allows separating different sampling systems from each other within different depth. Small submersible pumps allowed depth-specific sampling without cross-currents. A detailed description of the multilevel sampling system is given by Schirmer et al. 1995 (Schirmer et al., 1995).
The depth discrete investigation of geochemical and isotope parameters in Zeitz at well 23/00 were previously described by Stelzer et al. (2006b). The position of BACTRAP systems, water table and geological information of well 23/00 are shown in Fig. 3-1 and Fig. 3-2.

3.3.4 Preparation and incubation of *in situ* microcosms (BACTRAP®)

Bio-Sep® beads (K. Sublette, University of Tulsa, Tulsa, USA) were loaded with contaminants as carbon substrate for microorganisms. The spherical beads, 2 to 3 mm in diameter, consist of powdered activated carbon (PAC) incorporated within an aramid polymer matrix (Nomex®). The beads have an internal porosity of 75%, an internal surface area greater than 600 m^2 g^{-1}, and outer pores of 1-10 microns (Peacock et al., 2004; White et al., 2003). The beads were heated at 300 °C for 4 hours to remove organic residues and then 0.2 to 0.4 g of beads were filled in perforated Teflon® tubes. Glass wool was used as a plug to keep the beads inside the Teflon® tubes. The filled Teflon® tubes were autoclaved at 121 °C for sterilization and rehydration of the beads. The microcosms were loaded with the substrate (benzene or toluene) via gas phase under reduced pressure as described previously (Geyer et al., 2005). The substrate and the microcosms were placed in small

glass containers which were evacuated at 60 mbar. To allow uniform adsorption of the contaminants the Bio-Sep® beads were incubated for at least 48 hours. Contrary to the previous method, the vacuum was then released by filling oxygen free water into the glass container and the BACTRAPs were stored under anoxic water until deployment in the monitoring wells. The oxygen free water was used to fill the free pore space of the beads due to the vacuum release to keep the *in situ* microcosm system anoxic. The BACTRAPs for the multi level experiment in Zeitz were loaded with [$^{13}C_6$] benzene to a concentration of about 76 mg g^{-1} and for the metabolite experiment with [^{13}C]-α toluene to a concentration of about 50 mg g^{-1}. The BACTRAPs deployed in Gneisenau were loaded with uniformly labelled [$^{13}C_6$] benzene and [$^{13}C_7$] toluene to a concentration of 500 mg g^{-1}. In all experiments, BACTRAPs were loaded with non labelled benzene or toluene in similar concentration for control experiments. Material from these control experiments was used for molecular biological studies and to investigate the isotope composition of fatty acids at natural abundance.

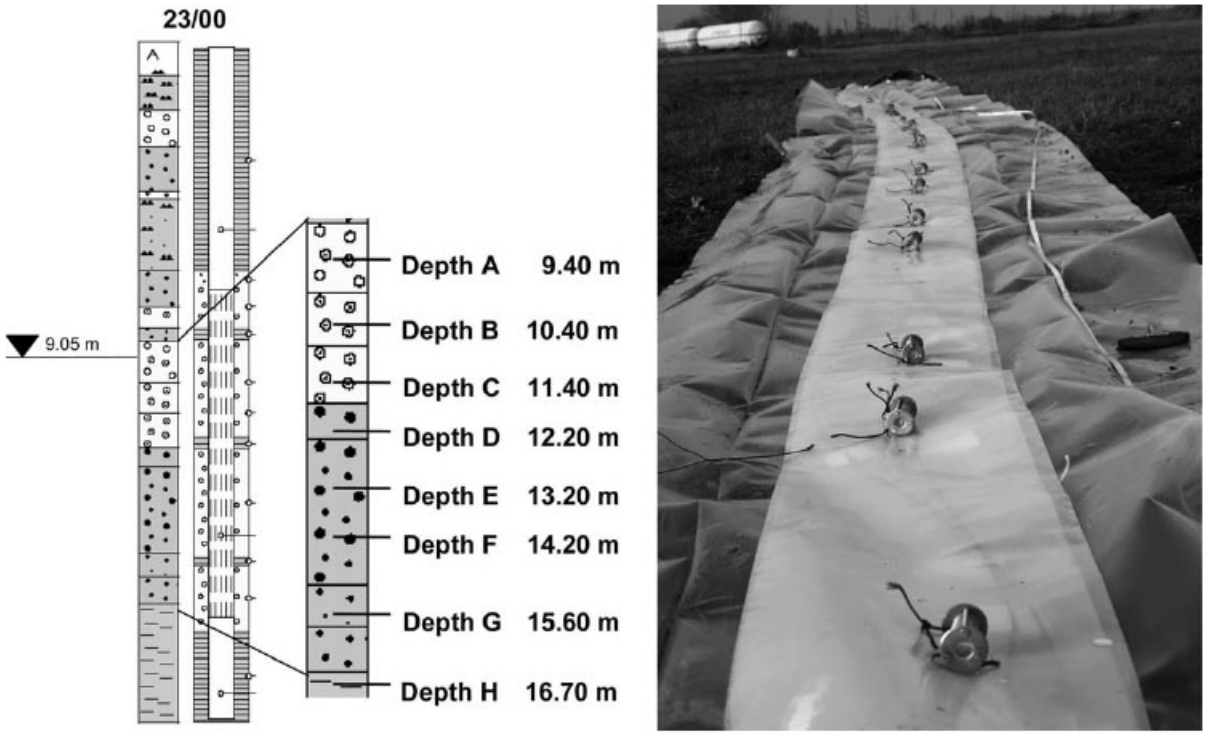

Fig. 3-2: Left: Geological profile at monitoring well 23/00 (Zeitz, Germany). The location of the filter screen is illustrated. The groundwater table was located at 9.05 m below ground. *In situ* microcosms were installed at A, C, D, F, G, H and samples for geochemical analyses were taken from all depths except A. Right: The picture shows the multi level sampling system with stainless steel containers for *in situ* microcosms before subjection into the well.

The microcosms were deployed with the MLPS or in monitoring wells over a time period of 51 to 100 days. In the MLPS experiments the microcosms were placed in stainless steel cages as a spacer to ensure a permanent flow of groundwater through the *in situ*

microcosms and to avoid contamination with biological material by direct contact with the packer membrane or the filter screen of the well.

3.3.5 Extraction and Derivatisation of Fatty Acids

The Bio-Sep® beads were extracted using a dichloromethane-methanol-water mixture as solvent modified from Bligh and Dyer 1959 (Bligh and Dyer, 1959). After phase separation the dichloromethane phase containing the fatty acids (FA) was evaporated to dryness and derivatised using a trimethylchlorosilane (TMCS) methanol mixture (1:8; v:v) as reactant for 2 h at 70 °C to obtain fatty acid methyl esters (FAME) (Thiel et al., 2001). After evaporation to complete dryness the FAME fraction was dissolved in *n*-hexane for subsequent analysis by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope-ratio-monitoring-mass-spectrometer system (GC-C-IRMS).

3.3.6 Analysis

GC-MS. For identification and structural characterisation by GC-MS a Hewlett Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent, Palo Alto, USA) was used. The carboxylic acid fraction was separated on a BPX-5 column (30 m*0.32 mm*0.25 µm) (SGE, Darmstadt, Germany) with a temperature program of 120 °C initial temperature for 4 min, heat at 4 °C min^{-1} to 250 °C, heat at 20 °C min^{-1} to 300 °C, and hold for 10 min. FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Sigma-Aldrich, Germany). The fatty acids are designated in the form of *A:BωC* where A is the number of carbon atoms, B is the number of double bonds and C is the distance of the closest double bond from the aliphatic end of the molecule (unsaturation, *ω*-nomenclature). The prefix *i* (*iso*) and *a* (*anteiso*) refer to methyl branching. The benzylsuccinic acid methyl ester was characterised by co-injection and comparison of mass spectra of the authentic reference compound which was derivatised as described above.

GC-C-IRMS. The carbon isotope composition of the carboxylic acids fractions was analysed using a GC-C-IRMS. The system consists of a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany) as described previously (Richnow et al., 2003a). A BPX-5 column (50 m*0.32 mm*0.5) (SGE, Darmstadt, Germany) was used for chromatographic separation of fatty acid methyl esters with helium as carrier gas at a flow rate of 1.5 mL min^{-1} and a temperature program with initial temperature of 60 °C for 2 min, heat at 20 °C min^{-1} to 120 °C, heat at 2 °C min^{-1} to 300 °C, and hold for 20 min (Miltner et al., 2004). The analysis of BTEX (Vieth et al., 2005) on the same system was described previously.

The carbon isotope ratio of fatty acids is reported in δ-notation (per mill) relative to the Vienna Pee Dee Belemnite standard (V-PDB) with known isotopic composition (Anonymus,

1995). The isotope composition of highly ^{13}C-enriched fatty acids could not be determined accurately. The instrument was calibrated to about 1000 δ units. Higher values are outside the calibration range of the instrument and the uncertainty increases with higher enrichments. Therefore, positive isotope compositions of fatty acids were only reported with the first 2 decimal places of the value.

3.3.7 Molecular biological analysis

For each sample, a total of 50 Bio-Sep® beads were transferred into 15 ml Falcon tubes containing 12 ml of TES buffer (50 mM $NaCl_2$, 10 mM Na_2EDTA, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride [Tris HCl], pH 8.0) with 1% sodium dodecylsulphate (SDS). Five cycles of freeze-thaw lysis were performed, each with 5 min in liquid nitrogen (-196 °C) and 5 min at 65 °C in a shaking water bath, each cycle was interrupted by 10 s of vigorous vortexing. DNA was then extracted with phenol-chloroform, as described elsewhere (Schwieger and Tebbe, 1998; Schwieger and Tebbe, 2000). The isopropanol precipitated and purified DNA of each tube was resuspended in a total of 40 µl of 10 mM Tris in 10 µl aliquots. Aliquots were kept at 4 °C for further analysis or stored at -20 °C.

Partial 16S rRNA genes were amplified from total DNA by PCR, using primers Com1 and Com2ph (Schmalenberger et al., 2001). The PCR products were converted to single strands by lambda-exonuclease digestion, following the protocol of Schwieger and Tebbe (1998). Genetic profiles of each sample were generated by single strand conformation polymorphism (SSCP) on non-denaturing polyacrylamide gels, as described in detail elsewhere (Dohrmann and Tebbe, 2004; Tebbe et al., 2001). Single bands were selected for further DNA-sequencing after PCR-amplification and cloning in Escherichia coli. Sequencing is described in the same references (Dohrmann and Tebbe, 2004; Peters et al., 2000; Tebbe et al., 2001). Consensus sequences were compared to database sequences using the FASTA tool provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta/). DNA-sequences of this study have been deposited in the GenBank database under the accession numbers AJ123456 to AJ123478.

3.4 Results and Discussion

3.4.1 Zeitz

In order to test the BACTRAP concept, the BTEX contaminated aquifer in Zeitz (Germany) was chosen as a model site, because detailed information was available about the contamination plume and the geochemical zones from previous studies (Fischer et al., 2005; Fischer et al., 2004; Vieth et al., 2001; Vieth et al., 2005). At well 32/02 (Fig. 3-1), located downstream of the contamination source and characterised by moderate benzene concentrations of approximately 44 mg L^{-1} in 2002, *in situ* microcosms supplemented with

^{13}C-labelled toluene and benzene provided evidence for the *in situ* degradation of these substances, as described in detail elsewhere (Geyer et al., 2005).

For further BACTRAP studies we selected well 23/00 (Fig. 3-1, Fig. 3-2) because of generally higher concentrations of contaminants and clear indication of microbial sulphate reduction as the predominant terminal electron accepting process by sulphur isotope analysis (Fischer et al., 2004).

A depth specific MLPS approach was chosen to investigate different geochemical zones of the aquifer within only one monitoring well (Fig. 3-2). The benzene plume showed a vertical structure at this monitoring well (Fig. 3-3). The benzene concentration increased from 415 µmol L^{-1} at 10.4 m below the surface, which was located near the water table at 9.05 m, to a maximum of more than 6000 µmol L^{-1} in the depth interval between 13 to 14 m. Towards the aquitard, located at about 17 m, the benzene concentration decreased to about 1700 µmol L^{-1} at 16.7 m depth.

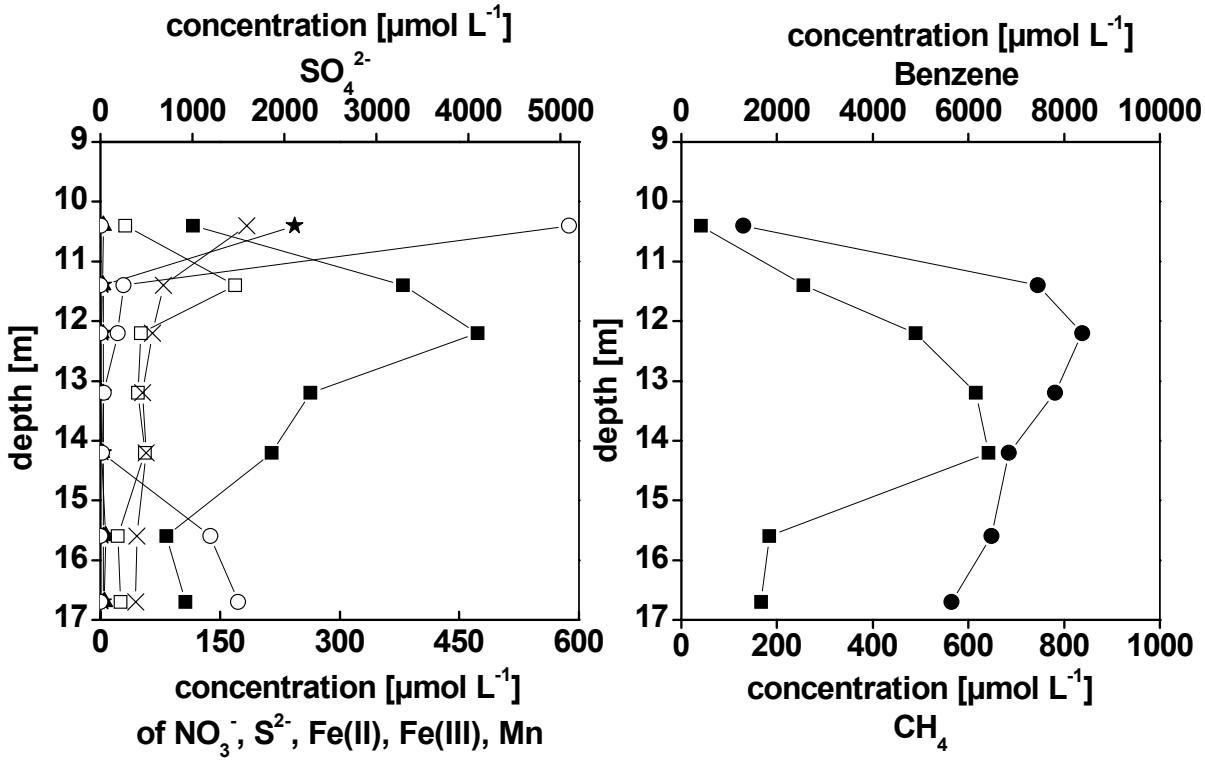

Fig. 3-3: Left: Concentration of sulphate (○), nitrate (*), sulphide (▲), ferrous iron (■), ferric iron (□) and manganese (x) in [µmol L^{-1}]. Right: benzene (■) and methane (●) concentration in [µmol L^{-1}] at Zeitz (Germany) monitoring well 23/00.

In uncontaminated zones of the aquifer upstream the plume, the concentrations of nitrate were relatively low (< 100 µmol) compared to sulphate concentrations (> 10 mmol) suggesting that nitrate was not a dominant electron acceptor in this aquifer. At the observation well manganese and nitrate were only present in the upper zone of the aquifer in elevated concentrations (Fig. 3-3). The low total manganese concentrations indicated that manganese mobilisation by Mn(IV) reduction was not an important electron accepting

process at the field site. Thus nitrate and manganese may act as potential electron acceptors at the water table close to the capillary fringe, where also some oxygen may enter from the gaseous phase of the soil into the aquifer system. Ferrous iron mobilisation suggested that ferric iron was a potential electron acceptor. However, the concentration of ferrous iron may not show the true extent of iron reduction, because ferrous iron can be precipitated in the presence of H_2S. However, as discussed elsewhere iron reduction is not an important terminal electron accepting process in this aquifer (Dethlefsen et al., 2004). Sulphate concentrations > 10 mmol L^{-1} are typical for uncontaminated parts of the aquifer (Fischer et al., 2004) and in the upper part of the vertical profile sulphate concentrations up to 5 mmol L^{-1} were found. The depletion of sulphate within the plume to concentrations of less than 0.2 mmol L^{-1} between 11.5 and 14.5 m suggested intensive microbial sulphate reduction although the development of sulphide was low. Sulphide may be precipitated by iron, which emphasises the difficulties to trace the degradation of contaminants by electron donor acceptor balances. Thus, sulphate was presumably the predominant electron acceptor.

Fatty acid composition. The fatty acid patterns extracted from the BACTRAPs were dominated by hexadecanoic (C16:0) and octadecanoic (C18:0) acids. Unsaturated hexadecenoic (C16:1) and octadecenoic (C18:1) acids were present in lower concentrations (Fig. 3-4). Other linear saturated fatty acids like tetra (C14:0), penta- (C15:0) and heptadecanoic (C17:0) acids as well as *iso* and *anteiso* branched fatty acids with 15 carbon atoms were present in much lower concentrations.

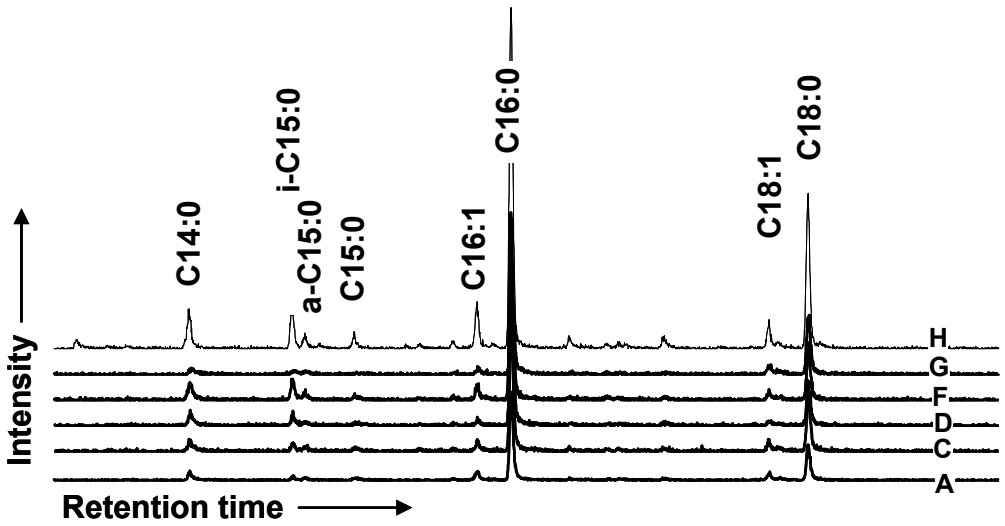

Fig. 3-4: The composition of the total fatty acid fraction (m/z 74) obtained from *in situ* microcosm experiment deployed at various depths (A to H) in well 23/00 at the field site Zeitz (Germany). The depths of the microcosm (A to H) and the position in the aquifer are shown in Fig. 3-2.

The *iso* and *anteiso* branched fatty acids are used as biomarkers for Gram-positive bacteria suggesting their involvement in biodegradation in some zones of the aquifer (Kaur et al., 2005). The fatty acid patterns of all depths were very similar although geochemical

conditions were changing, suggesting that the fatty acid pattern did not respond sensitively to the geochemical conditions. However, the composition of fatty acids found in benzene amended *in situ* microcosms at marginal part of the plume was more diverse suggesting a more diverse microbial community (Geyer et al., 2005). Further biomarker fatty acids specifically characterising some individual sulphate reducing bacterial strains were not observed (Kaur et al., 2005). In our studies we could not use the fatty acid pattern for a taxonomic interpretation.

Isotope signature of fatty acids. The isotope compositions of fatty acids ($\delta^{13}C_{FA}$) extracted from the BACTRAPs amended with [$^{13}C_6$] benzene were between -28 ‰ and 439 ‰ (PDB) (Tab. 3-1). The variation in isotope composition may indicate the presence of organisms feeding on various carbon sources. Fatty acids with an isotope signature lower than -25 ‰ show the typical natural abundance of $\delta^{13}C_{FA}$ found in soil and aquifer materials (Pelz et al., 2001a; Pelz et al., 2001b) and are typical for organisms using natural occurring non labelled carbon sources. In BACTRAP experiments with non labelled substrate or without any substrate supply via Bio-Sep® beads all fatty acids showed natural abundances (-35 to -20 ‰, data not shown). Fatty acids with a positive carbon isotope signature (>0 ‰) clearly indicated that microorganisms incorporated the labelled carbon from the provided [$^{13}C_6$] benzene (Tab. 3-1). The enrichment of ^{13}C in fatty acids can only stem from the labelled substrate and thus clearly provided evidence of *in situ* benzene degradation.

Tab. 3-1: Carbon isotope composition of fatty acids extracted from *in situ* microcosms incubated with $^{13}C_6$ labelled benzene at monitoring well 23/00 (Zeitz, Germany) for 6 weeks. A to H refers to incubation depth indicated in Fig. 3-2.

depth	$\delta^{13}C_{FAME}$ [‰]							
	C14:0	i-C15:0	a-C15:0	C15:0	C16:1	C16:0	C18:1	C18:0
A	63	89	-22	-18	330	62	22	-21
C	-8	90	-19	-22	439	47	61	-15
D	-20	-17	-28	-23	35	-13	-21	-24
F	-17	1	-23	-23	86	15	-2	-25
G	-22	134	-23	-20	174	20	-6	-24
H	-22	-13	-22	-26	24	-3	-16	-26

BACTRAPs with ^{13}C-labelled benzene incubated in different geochemical zones showed variations in the ^{13}C-enrichment of fatty acids within the various depths although the composition of fatty acids did not change significantly. Hexadecenoic acid (C16:1) showed the highest incorporation of ^{13}C (439 ‰) next to i-C15 (134 ‰) and C16:0 (62 ‰). a-C15, C15:0, C14:0 and C18:0 displayed only low or even no incorporation of ^{13}C. The i-C15 generally showed a significantly higher labelling than a-C15 which was almost not labelled. The variation in $\delta^{13}C_{FA}$ clearly indicated that the organisms producing these FA grew on different carbon sources. Fatty acids displaying a higher incorporation of ^{13}C were very likely

produced by organism feeding on [$^{13}C_6$] benzene while fatty acids from bacteria with lower or no ^{13}C incorporation were likely not involved in the degradation of [$^{13}C_6$] benzene. Cross feeding by metabolites may channel labelled carbon into individual members of the microbial community.

In the upper zone of the aquifer the ^{13}C incorporation was higher, which may suggest a more intensive transformation of the [$^{13}C_6$] benzene. This might be a result of more attractive electron acceptors for degradation such as nitrate, manganese and iron as well as oxygen close to the water table. Nevertheless, also in the depth between 12 and 14 m indications for benzene degradation were found despite high benzene concentrations. Here sulphate reducing conditions were likely present.

On the other hand, labelling of biomass can be influenced by the availability of labelled benzene on the BACTRAPs relative to the unlabelled benzene from the groundwater itself. The concentration of benzene in the upper part of the plume was lower compared to deeper zones of the aquifer (Fig. 3-3). Therefore, benzene derived from the contamination might be available as a carbon source to a relatively higher amount in the depth of 12 and 14 m with benzene concentration up to 6 mmol L^{-1} (Fig. 3-3). The relative intensity of ^{13}C incorporation into biomass changed more pronounced with depth than the composition of the fatty acid fraction and might therefore reflect the relative intensity of benzene metabolisation and geochemical conditions. Compared to previous investigations with [$^{13}C_6$] benzene amended *in situ* microcosms at marginal parts of the plume where fatty acids were labelled up to 13000 ‰ (Geyer et al., 2005), the ^{13}C incorporation found in the multi level investigation was significantly lower (max. 439 ‰) suggesting a less intensive metabolisation at the different depth of this monitoring well.

3.4.2 Gneisenau: Upper quaternary aquifer

The compositions of the total fatty acid fractions obtained from *in situ* microcosms loaded with [$^{13}C_6$] benzene and [$^{13}C_7$] toluene were compared in order to investigate the microbial communities growing on these different substrates. The *in situ* microcosms were exposed to the upper quaternary aquifer Gneisenau, Germany (P10F) over 12 weeks and analysed for composition and isotope signatures of fatty acids. The patterns of fatty acids derived from toluene and benzene amended BACTRAPs incubated in the source zone of the Quaternary aquifer (P10F) were almost identical (Fig. 3-5). C18:0, C18:1 and C16:0 were dominating the fatty acid patterns. C18:1, *iso* and *anteiso* branched C15 compounds as well as C15:0 and C14:0 were present in minor concentrations. The similarity in fatty acid compositions of both the benzene and toluene amended BACTRAPs (Fig. 3-5) indicated that the substrate did not affect the pattern of fatty acids suggesting that the lipid pattern is not a sensitive indicator to determine changes in the microbial community.

However, the isotope composition of the fatty acids from the benzene amended microcosm incubated in well P10F showed drastic differences in comparison to the toluene loaded one (Tab. 3-2). Only the hexadecanoic acid (C16:0) of the [$^{13}C_6$] benzene amended BACTRAP were found to be labelled. Unsaturated C18:1 and C18:2 isomers were only slightly or almost not labelled. The concentrations of other fatty acids were too low for the determination of their isotope composition (Fig. 3-5, Tab. 3-2). In contrast, the [$^{13}C_7$] toluene amended BACTRAP of well P10F showed a very strong incorporation of ^{13}C into fatty acids with carbon numbers between C14 to C18 of which hexadecenoic acid (C16:1) was most intensively labelled (45000 ‰). Interestingly the fatty acids with 18 carbon atoms were labelled two orders of magnitude lower. The significant high labelling of fatty acids from the toluene amended BACTRAP demonstrated extensive transformation of toluene by some community members whereas benzene carbon was only assimilated to a limited extent. Obviously, other members of the community, producing the significantly lower labelled fatty acids, used other carbon sources in addition to the labelled contaminants.

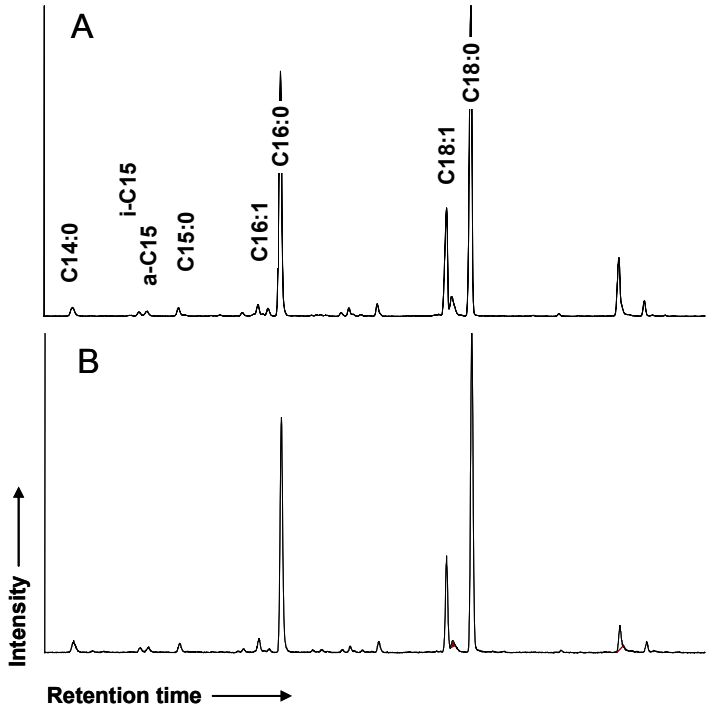

Fig. 3-5: Comparison of the composition of the total fatty acid fraction (m/z 74) obtained from *in situ* microcosms loaded with [$^{13}C_6$] benzene (A) and [$^{13}C_7$] toluene (B). The *in situ* microcosms were both exposed in the upper quaternary aquifer (P10F) at the field site Gneisenau (Germany) over 12 weeks.

3.4.3 Gneisenau: Lower cretaceous aquifer

In situ microcosms were also applied to evaluate the biodegradation of benzene and toluene in the deeper fractured cretaceous aquifer. This aquifer was a hundred times higher contaminated with benzene (up to 45 mg L^{-1}) than the upper quaternary aquifer. BACTRAPs

loaded with [$^{13}C_6$] benzene, [$^{13}C_7$] toluene and with non labelled substrates were exposed up stream the source zone (P8T) and in the centre of the plume (P11T). A higher enrichment of ^{13}C in fatty acids, e.g. in C16:1 (96000‰) and C16:0 (9700 ‰), was only observed in the toluene amended microcosms up stream the source area (P8T) in relatively uncontaminated zones of the deeper fractured aquifer (Tab. 3-2). The fatty acids of benzene or toluene amended BACTRAPs from the source zone (P11T) in the deeper aquifer were only slightly labelled in both cases (Tab. 3-2). The fatty acids occurring in relatively higher concentrations such as C16:0, unsaturated C18 isomers and C18:0 were almost not labelled in comparison to fatty acids commonly present in lower concentrations such as C14-, C15-species and C16:1 (Tab. 3-2). This suggests that only a minor part of the community was involved in the contaminant degradation.

The results show that the transformation of contaminant derived carbon into fatty acids was higher in toluene compared to benzene amended BACTRAPs, which is in accordance to earlier findings that toluene is more easily degradable under anaerobic conditions than benzene (Cunningham et al., 2001; Da Silva and Alvarez, 2004; Wiedemeier et al., 1999). In a direct comparison of parallel experiments with labelled toluene and benzene, the amount of ^{13}C transferred into fatty acids may be used as a direct indicator for the *in situ* degradability of the substrates. The highest enrichment of ^{13}C into fatty acids was observed in BACTRAPs loaded with labelled toluene indicating a higher degradation capacity for toluene compared to benzene. As the *in situ* microcosms were exposed in an almost uncontaminated zone of the deeper aquifer a significant potential to degrade the contaminants was present even in almost non-contaminated zones.

Tab. 3-2: Carbon isotope composition of fatty acids extracted from *in situ* microcosms incubated with $^{13}C_6$ labelled benzene and $^{13}C_7$ labelled toluene in the upper Quaternary monitoring well (P10F) and in the lower Cretaceous monitoring wells (P8T, P11T) at the field site Gneisenau (Germany) for 12 weeks.

	$\delta^{13}C_{FAME}$ [‰]				
well	**P8T**	**P10F**		**P11T**	
Fatty Acid	**^{13}C-Toluene**	**^{13}C-Benzene**	**^{13}C-Toluene**	**^{13}C-Benzene**	**^{13}C-Toluene**
14:0	n.d.	n.d.	2800	n.d.	390
i-15:0	41000	n.d.	14000	n.d.	30
a-15:0	n.d.	n.d.	1800	n.d.	-21
15:0	n.d.	n.d.	580	172	n.d.
i-16:0	n.d.	n.d.	7600	n.d.	-42
16:1	96000	n.d.	46000	n.d.	140
16:0	9700	9	3600	-9	-18
18:2-18:1-Cluster	400	-17	540	-16	-19
18:0	3	-27	290	-30	-26

n.d. not determined due to low concentration

To sum up the results, Bio-Sep® beads spiked with ^{13}C BTEX can be used to investigate the *in situ* biodegradation of BTEX compounds semi-quantitatively by the amount of incorporated ^{13}C in lipid derived fatty acids. The intensity of ^{13}C incorporation into biomass (FA) provides valuable information on microbial diversity and on the relative amount of metabolisation. The quantification of biodegradation was not possible in these experiments, but with an absolute quantification of fatty acids and an estimation of the proportion of fatty acids in anaerobic bacteria it may be possible to quantify the incorporation into the biomass. Thus further development is needed to apply the BACTRAP system to quantify *in situ* biodegradation.

3.4.4 Molecular biological analysis of microbial communities on BACTRAPs

In order to characterise the contaminant degrading microbial community in the aquifer the biodiversity of the microbial biomass can be analysed by cultivation independent molecular biological approaches based on PCR-amplified partial 16S rRNA genes from total DNA extracted from groundwater samples (Scow and Hicks, 2005). In the present study, total DNA was extracted from the BACTRAPs and the PCR products amplified from the different samples (wells) were compared to each other by their SSCP profiles (Fig. 3-6). This technique is now widely used in microbial ecology to compare the structural diversity of microbial communities (Dohrmann and Tebbe, 2004). In addition to comparing the structural diversity of the bacterial community from different samples, bands of the SSCP-profiles can be characterised by DNA-sequencing in order to identify individual members of the bacterial community (Dohrmann and Tebbe, 2004).

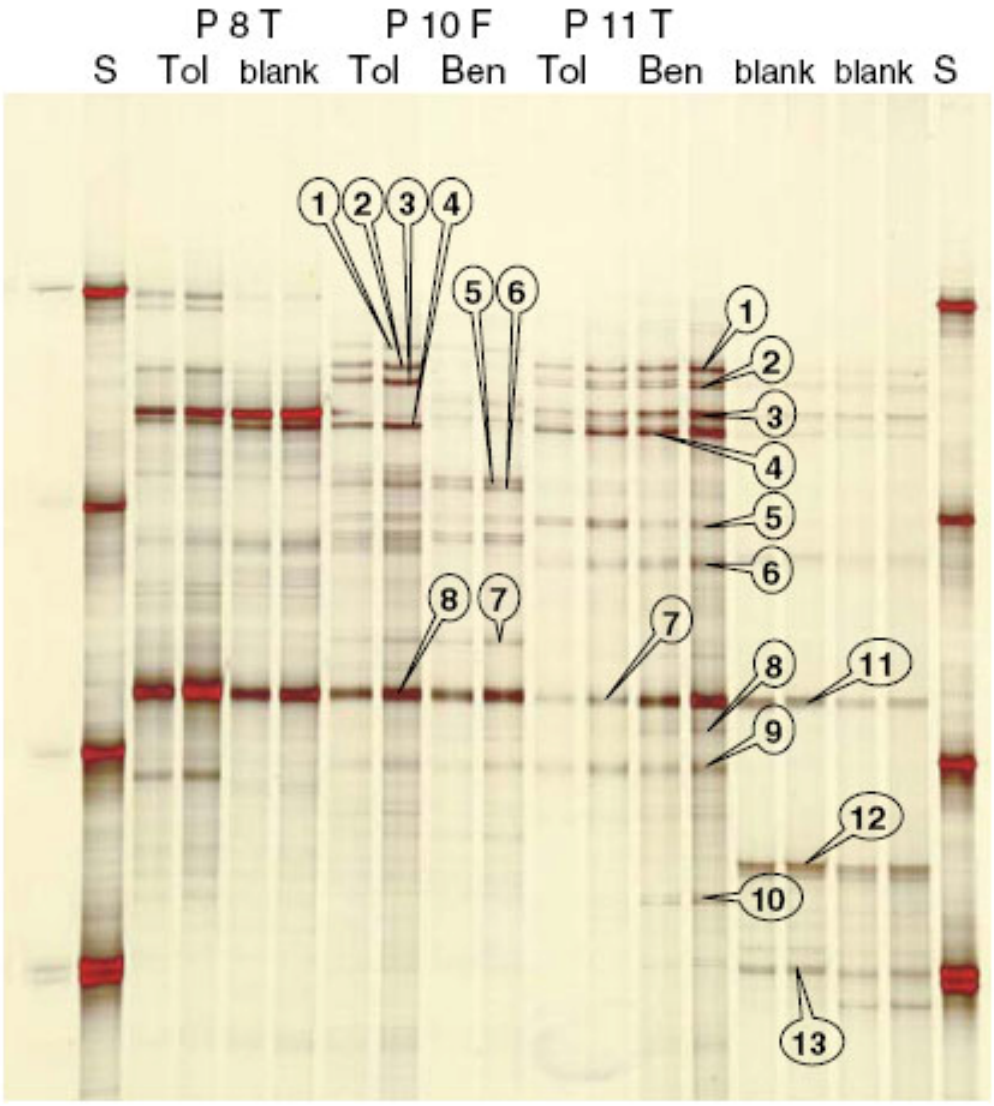

Fig. 3-6: SSCP Gel of PCR amplified 16S rDNA isolated from BACTRAPs loaded with $^{13}C_7$ toluene or $^{13}C_6$ benzene after exposure (84 d) in a contaminated aquifer. The bands (numbers) are sequenced for identification (see Tab. 3-3).

Tab. 3-3: Characterization of rRNA genes isolated from BACTRAPs incubated in the lower (P11T) and upper (P10F) aquifer at the field site Gneisenau (Germany).

Band	Clone	Length [bp]	Identity [%]	Closest relative in database (GenBank Accession No.)	Phylogenetic group	Source of sequence or isolate
Upper aquifer (P10F)						
1	P10F-01c	371	99.7	uncultured bacterium (AF534262)	Deltaproteobacteria	marine and freshwater sediments
1	P10F-01a	368	99.5	uncultured bacterium (DQ404728)	Candidate division OP3	contaminated sediment
2	P10F-02b	371	98.4	uncultured bacterium (AF050590)	Firmicutes	hydrocarbon- and chlorinated-solvent-contaminated aquifer
2	P10F-02b	374	99.5	uncultured bacterium (AY050586)	Deltaproteobacteria	monochlorobenzene contaminated groundwater
3	P10F-03c	371	97.6	unidentified bacterium (AF058010)	Deltaproteobacteria	PCB-dechlorinating enrichment culture
4	P10F-04c	369	98.9	uncultured bacterium (AY475201)	Alphaproteobacteria	metal-rich and acidic river water
6	P10F-06b	369	100	*Ralstonia pickettii* (AY741342)	Betaproteobacteria	clinical origin
7	P10F-07a	367	99.7	uncultured bacterium (AJ306737)	Bacteroidetes	1,2-Dichlorpropane dechlorinating consortium
8	P10F-08b	369	98.9	uncultured bacterium (AB205680)	Alphaproteobacteria	denitrifying activated sludge
Lower aquifer (P11T)						
1	P11T-01a	368	97.3	uncultured bacterium (DQ404728)	Candidate division OP3	contaminated sediment
1	P11T-01b	371	99.4	*Geobacter pelophilus* (U96918)	Deltaproteobacteria	dissimilatory Fe(III)-reducing bacteria
2	P11T-02a	371	98.7	uncultured bacterium (AY945913)	Betaproteobacteria	quinoline-removal bioreactor
3	P11T-03a	371	99.2	uncultured bacterium (AF104274)	Deltaproteobacteria	benzene-contaminated aquifer sediments
5	P11T-05b	369	99.2	uncultured bacterium (AB240520)	Betaproteobacteria	rhizosphere biofilm of *Phragmites* at river
5	P11T-05a	369	100	*Ralstonia pickettii* (AY741342)	Betaproteobacteria	clinical origin
6	P11T-06d	371	98.4	uncultured bacterium (AY250093)	Betaproteobacteria	napthalene-contaminated sediment
6	P11T-06c	367	97.3	uncultured bacterium (AB240238)	Bacteroidetes	reed bed reactor
7	P11T-07c	369	99.2	uncultured bacterium (AB205680)	Alphaproteobacteria	denitrifying activated sludge
9	P11T-16b	366	92.3	uncultured bacterium (AB205753)	Firmicutes	denitrifying activated sludge
10	P11T-10b	367	99.4	uncultured bacterium (AJ519404)	Bacteroidetes	uranium mining waste pile
13	P11T-14d	371	88.9	uncultured bacterium (UEU81707)	Chlamydiae	anaerobic digester

Most of the SSCP profiles generated in this study were relatively similar to each other, indicating a reproducible colonisation pattern of the bead material independently of the geochemical conditions in the investigated groundwater wells (Fig. 3-6). For most samples analysed, the bacterial community compositions on the benzene and toluene loaded microcosms were only slightly altered in comparison to the non-amended ones (blank). However, the fact that highly labelled microbial fatty acids were extracted from the ^{13}C experiments indicated a substrate degradation and incorporation into biomass to a different extent (Tab. 3-2). This may show a coordinated degradation by certain members of the microbial community colonising the *in situ* microcosms and a channelling of the ^{13}C carbon into the microbial food web. However, even though some fatty acids were highly labelled, the isotope composition showed that these organisms used also other carbon sources. In the highly contaminated part of the aquifer, benzene and toluene were presumably the major carbon sources but other BTEX compounds and minor contaminants as well as humic substances were certainly present and may be used as carbon sources as well. In addition, some fatty acids in ^{13}C experiments were not labelled and indicated that microbes not involved in pollutant degradation were also colonising the bead material.

The comparison of the non-amended (blank) with the loaded BACTRAPs showed that the SSCP- and FA patterns were only slightly different (Fig. 3-5, Fig. 3-6). This reflects the fact that the bead material (activated carbon) of the blanks extracted a certain amount of the contaminant i. e. benzene from the contaminated aquifer leading to similar conditions in the non loaded microcosms compared to the benzene loaded ones. The SSCP- and FA patterns from the toluene loaded BACTRAPs were also only slightly different from the non-amended (blank) and benzene BACTRAPs showing that toluene degraders were members of the colonising community.

Detailed analyses showed that the profiles from the different wells clustered together mostly independent from the loaded substrates (data not shown). This indicated a dominant effect of the environmental conditions of each well on the communities developing on the BACTRAPs and a minor influence of the supplied substrate. Therefore, no signals with respect to new emerging bands showed the colonisation of specific contaminant degraders on the loaded *in situ* microcosms. The intense bands of the patterns from the low contaminated area (P8T) indicated a good growth on the microcosms whereas the generally smaller bands from the wells of the contaminated area (P10F and P11T) showed lower biomass accumulation and may point to a certain toxicity of the benzene in that area (Fig. 3-6). The identified DNA-sequences of the SSCP-profiles were mostly related to sequences from yet uncultivated bacterial species (Tab. 3-3, Fig. 3-7). Phylogenetic analyses revealed a community dominated by iron and sulphate reducing bacteria particularly of the *Geobacter* and *Desulfuromonas* group (Lovley and Phillips, 1988; Zhang et al., 2003). These groups of

organisms are known to be involved in contaminant degradation in anaerobic aquifers. This shows that iron and sulphate reducing microbial communities are likely to be involved in the contaminant degradation and that iron and sulphate were used as terminal electron acceptors at this field site.

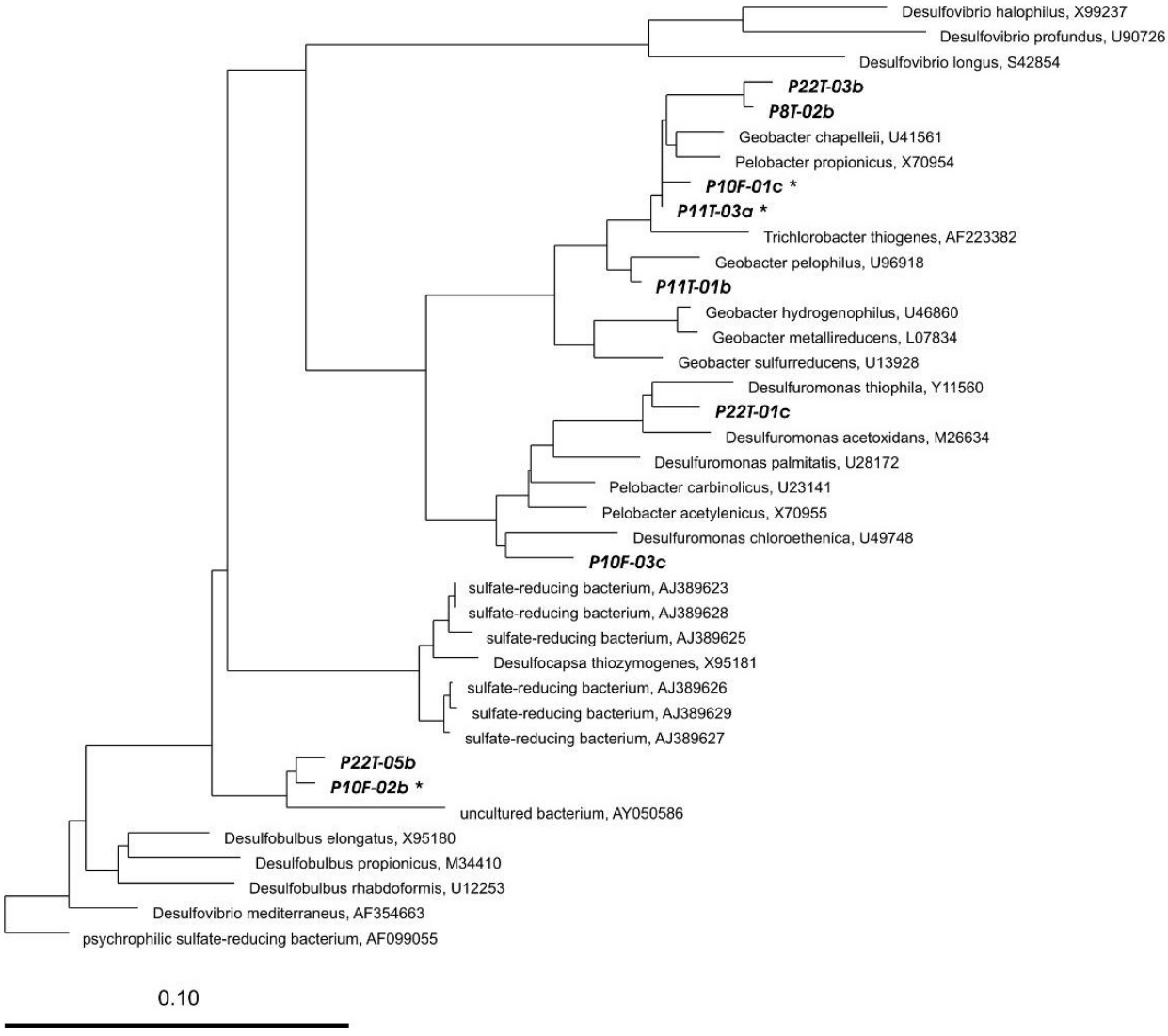

Fig. 3-7: Phylogenetic analysis (maximum likelihood method) of PCR-amplified partial 16S rRNA genes from the group Deltaproteobacteria, retrieved from BACTRAPs exposed at the field site Gneisenau. Clones are indicated by their source of isolation (P10F for upper and P11T for lower aquifer) followed by their clone number. * indicates sequences which were found at two subsequent sampling dates in the same well.

The molecular biological data supported the results of the hydrological and geochemical analyses and provided valuable information about the biodiversity of the degrading microbial community. In addition, the BACTRAP approach with labelled contaminants provided an excellent tool for assessment and proving the microbial *in situ* activity in contaminated aquifers. However, the key organisms related to contaminant degradation could not be identified. In future, growth experiments to investigate the kinetic of the colonisation traced by quantitative PCR may elucidate the development of communities and may provide

indications for organisms supported by the test substrate. Provided that the total biomass on BACTRAPs is sufficient, stable isotope probing (SIP) may also be applicable to identify organisms which used the ^{13}C labelled contaminants primarily as a carbon substrate (Lueders et al., 2004; Manefield et al., 2004).

3.4.5 Investigation of metabolic pathways

The extracted carboxylic acid fraction from *in situ* microcosm experiments with [^{13}C]-α-toluene contained ^{13}C labelled metabolites which could be easily identified by GC-C-IRMS analysis, due to the very strong signal caused by the ^{13}C labelled carbon although their absolute concentration was very low (Fig. 3-8). An examination of the labelled peak by GC-MS gave a spectrum indicative for benzylsuccinic acid dimethyl ester (Fig. 3-9).

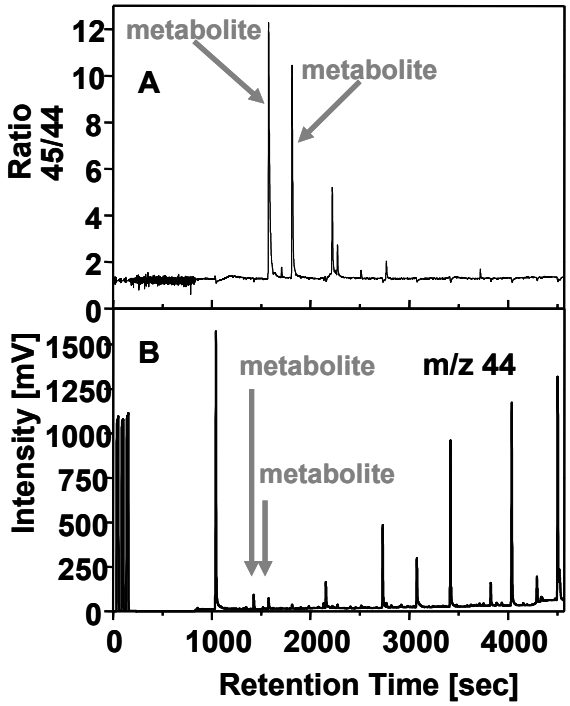

Fig. 3-8: GC-C-IRMS-chromatogram of the carboxylic acid fraction extracted from a [^{13}C]-α-toluene amended BACTRAP incubated at the field site Zeitz (Germany). The metabolites gave very strong signals in the 45/44 ratio plot (A) although their absolute concentration displayed by the amount of CO_2 (m/z 44) was relatively low (B).

The non labelled analogue was found in the carboxylic acids fraction of the BACTRAPs amended with non labelled toluene. The presence of benzylsuccinate was further confirmed by comparison of the mass spectra as well as the retention time of the derivatized authentic standard. The formation of benzylsuccinate is indicative for the anaerobic toluene degradation pathway which is employed by sulphate and iron reducing microorganisms able to degrade toluene (Heider et al., 1999; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Benzylsuccinate has been used as an indicator for anaerobic toluene degradation in

contaminated aquifers before (Beller, 2000; Griebler et al., 2004b). Therefore, the benzylsuccinic acid extracted from the *in situ* microcosm is a good indicator for the biogeochemical conditions governing the toluene degradation in this particular aquifer.

This clearly shows that BACTRAPs can also be useful to identify degradation pathways in the aquifer by analysing the potential metabolites. Reusser and Field (2002) applied push and pull experiments with deuterium labelled toluene to verify *in situ* toluene degradation in a contaminated aquifer. Although deuterium labelled contaminants may be useful tracer compounds to provide evidence for *in situ* degradation, much higher amounts of labelled substances are needed during push and pull experiments in comparison to the BACTRAP approach. As described here, the application of *in situ* microcosms can significantly reduce the amount of labelled material and gives in principle similar information as obtained in push and pull experiments. When using ^{13}C labelled compounds, the transformation into biomass can be traced by analysing the isotope composition of fatty acids and perhaps may also provide kinetic information in future.

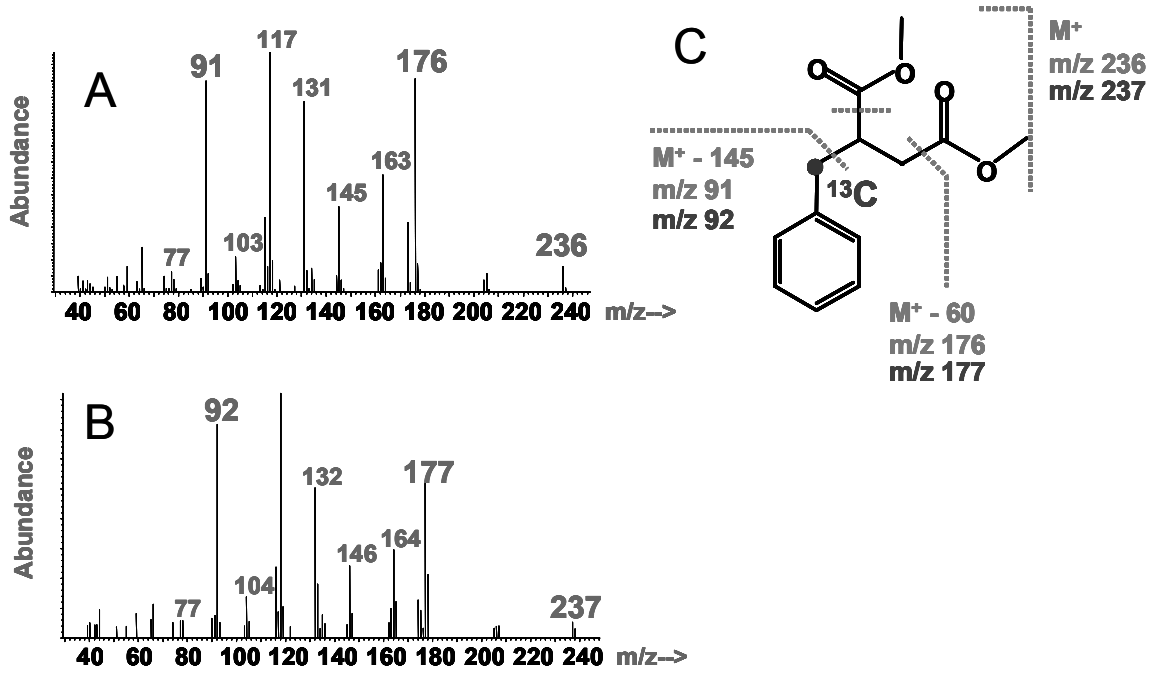

Fig. 3-9: Mass spectra of benzylsuccinate extracted from *in situ* microcosms incubated 92d within an anaerobic aquifer at the field site Zeitz (Germany). (A) BACTRAP loaded with non labelled toluene, (B) BACTRAP loaded with [^{13}C]-α-toluene. The fragmentation pattern of ^{13}C labelled benzylsuccinate shows that the ^{13}C atom was found at the position of the former methyl group (C).

3.5 Conclusions

In situ microcosms with ^{13}C labelled substrate can be used to demonstrate *in situ* biodegradation of hardly degradable contaminants such as benzene and toluene under anoxic conditions by analysing the isotope composition of fatty acids extracted from the biomass grown on the BACTRAP. The isotope composition in combination with the pattern of

fatty acids indicated that a complex microbial community was colonising the BACTRAPs, but apparently not all community members were involved in the contaminant degradation. The fatty acid patterns were not of taxonomic value and no sensitive indicator to determine changes in the microbial community within various environmental conditions but isotope composition was very useful to provide evidence for *in situ* degradation. Molecular biological analyses showed that mainly uncultured iron and sulphate reducing communities were colonising the BACTRAPs which reflected the biogeochemical conditions and terminal electron accepting processes in the aquifer. By analysing the mass spectra of metabolites we are able to identify the *in situ* degradation pathways. Benzylsuccinate found on toluene amended BACTRAPs indicated that toluene was degraded via the benzylsuccinate pathway which is typical for sulfidogenic and iron reducing conditions.

In summary, BACTRAPs give qualitative information on *in situ* biodegradation of contaminants. Further more with additional molecular biological methods we were able to identify the structure of the microbial community growing on the BACTRAPs which may reflect the *in situ* microbial community to some extent. Compared to laboratory microcosm studies, the advantage of the BACTRAPs is that the incubation takes place under environmental conditions directly in the aquifer at the field site. At present, mass balances were not possible in BACTRAP experiments which is certainly a limitation compared to closed laboratory systems.

In terms of the assessment of *in situ* biodegradation in contaminated aquifers *in situ* microcosms with labelled substances are relatively simple, low-cost assays to gain information on the potential of the microbial community able to degrade contaminants within a reasonable timeframe under *in situ* conditions.

3.6 Acknowledgements

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4 Assessment of Microbial *In Situ* Activity in Contaminated Aquifers

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4.1 Summary

Microbial ecologists and environmental engineers share the interest in identifying the key microorganisms responsible for compound turnover in the environment and in estimating the respective transformation rates. For successful application of Natural Attenuation processes, a reliable assessment of the *in situ* turnover of a contaminant in an aquifer is essential. Here, we review and present new details of two recently developed approaches concerning the assessment of *in situ* biodegradation: a) determination of biodegradation in a contamination plume by stable isotope fractionation analysis (SIFA) caused by microbial metabolism and b) determination of the actual degradation under the respective environmental conditions in the aquifer by using *in situ* microcosms (BACTRAP®) amended with ^{13}C-labeled substrates as tracer compounds. Based on stable isotope fractionation analysis, the degradation occurring under anoxic biogeochemical conditions at a respective site can be calculated for the entire plume. This has been shown for benzene and toluene at the Zeitz site and partly for chlorobenzene at the Bitterfeld site. By use of the *in situ* microcosm approach with ^{13}C-labeled compounds, the microbial *in situ* degradation under strictly anaerobic conditions could be proven for benzene and toluene in Zeitz and for chlorobenzene in Bitterfeld. The transformation of ^{13}C-carbon of the labeled substrate into microbial fatty acids proved the assimilation of the pollutant resulting in the formation of biomass. In addition, metabolites such as benzylsuccinic acid were found in the toluene- amended microcosms indicating anaerobic degradation of toluene. This result corresponds to the geochemical conditions found at the field site and therefore, the microcosm approach with ^{13}C-labeled compounds can be used to assign the predominant *in situ* degradation pathways in a contaminated aquifer. Since fatty acids profiles alone are often too unspecific for a community analysis at species level, the composition of the microbial communities were analyzed by genetic profiling and sequencing of partial 16S rRNA genes PCR-amplified from total DNA extracted directly from the microcosms. Sequences retrieved from the microcosms indicated a dominance of not yet cultivated bacteria. Several sequences were phylogenetically closely related to sequences of bacteria known to be iron and sulfate reducers, typically found at sites polluted with BTEX and/or mineral oil. The results show that the current methods for

monitoring microbial *in situ* activity at present stage are valuable tools for improving environmental control of compound turnover and will speed up engineering approaches.

4.2 Introduction

Since Winogradski´s column experiments, microbiologists and biochemists have long tried to link the transformation of compounds in the environment with the key microorganisms involved in the respective transformation. This linking is a crucial prerequisite for shedding light into the black box of real world microbial processes. From a technical perspective, it is also necessary to have a detailed knowledge about the key processes for purposes of control and successful engineering. In the past decade, methodological advances in linking the structure of microbial communities with the actual activity and function of their members generated dramatic progress in the knowledge of microbial mediated environmental processes and led to the development of new innovative approaches such as waste water treatment using the Anammox process (Schmidt et al., 2003).

The fate of pollutants in contaminated aquifers is mainly governed by microbial degradation. Abiotic processes such as dispersion, dilution, sorption, and volatilization may contribute to a decrease in concentration but do not lead to a significant reduction of the mass of contaminants. Therefore, the evaluation of *in situ* biodegradation is essential for the implementation of Natural Attenuation (NA) concepts in ground water management strategies (US-EPA, 1999). Plate counting methods were used in the past for assessment of the number of microbes present; however, these methods only provide information about the cultivable species. Advanced conventional methods apply the laborious quantification of electron balances of typically complex electron donor - terminal electron acceptor interactions. Calculations then relate the decreasing contaminant concentrations to the consumption of electron acceptors in order to assess biodegradation processes (Clement et al., 2002). When tracing the fate of single compounds in contaminant mixtures, this approach is complicated particularly on the field scale in the very heterogeneous natural environment where several electron acceptor-donor interactions compete. Laboratory microcosm studies are often used to obtain information on *in situ* biodegradation. However, this approach is not reliable because the majority of microorganisms have not yet been cultivated (Ringelberg et al., 1997; Vestal and White, 1989). In particular, the reproducible cultivation of anaerobic bacteria that degrade typical contaminants such as BTEX and PAHs can be difficult and may led to misinterpretation of the intrinsic biodegradation potentials. Laboratory microcosm studies or percolation column experiments, simulating the contaminant degradation under controlled water flow conditions, are time-consuming; furthermore, the conditions in the laboratory are mostly different to the natural environmental conditions resulting in certain limitations in the reliability of the data acquired.

Recently, the ^{13}C-enrichment of phospholipid-derived fatty acids (PLFA) related to the consumption of ^{13}C-labeled substrates was used to characterize bacterial toluene degradation in soil, sediment or aquifer microcosms (Hanson et al., 1999; Pelz et al., 2001a; Pelz et al., 2001b) and to trace the assimilation of toluene along a food chain (Mauclaire et al., 2003; Pombo et al., 2002). The ^{13}C-tracer concept can provide useful information on carbon fluxes, metabolites and microbial food webs. At the field scale, microbial incorporation of ^{13}C-labeled acetate into biomarker molecules such as PLFA and DNA was successfully assessed to indicate microbes which were suggested to be responsible for the reduction of uranium(VI) (Chang et al., 2005). A mesocosm was designed to monitor the *in situ* dynamics of the microbial community in a BTEX polluted aquifer (Hendrickx et al., 2005). In this study, uncontaminated aquifer material was incubated either in the uncontaminated area or in a contaminated area of the aquifer located nearby and the developed bacterial communities were analyzed by molecular biology techniques. Other authors applied the electron donors glucose and ethanol to acidic uranium and nitrate contaminated groundwater. Fortunately, they were able to show the increase of 16S rRNA gene sequences related to previously cultured metal reducing δ-proteobacteria by 1-2 orders of magnitude using quantitative PCR techniques and time resolved sampling at the site (North et al., 2004). These approaches provide valuable information on the functional biodiversity in soils and sediments. However, from a perspective of the contaminant history they do not provide reliable information on the compound turnover actually occurring under *in situ* conditions in an aquifer. In addition, it is difficult to provide any predictive data on the future fate of the residual contaminants under varying environmental conditions. These approaches may even fail, if organisms with necessary physiological properties were not cultured previously, or if the physiological capacities of the respective organisms are not generally expressed even though the organisms are present in the contaminated aquifer. Such effects are known from *Dehalococcoides* sp. (Holscher et al., 2004; Kube et al., 2005).

The objective of the present work is to review the recently developed approaches focusing on the assessment of the *in situ* biodegradation in contaminated aquifers. Two approaches will be described dealing with a) determining the cumulated biodegradation in a contamination plume by use of fractionation of stable isotopes at natural abundance during microbial metabolism and b) the assessment of the actual degradation under the respective environmental conditions in the aquifer. The latter approach employs *in situ* microcosms (BACTRAP®) amended with ^{13}C-labeled substrates such as toluene, benzene, and monochlorobenzene and enables the reliable quantification of the actual degradation. It will also enable the identification of the microbial key players in the near future. We present new results from applying the two approaches at field sites.

4.3 Materials and methods

4.3.1 Chemicals

The chemicals and solvents were obtained in p.A. quality from Merck unless otherwise stated. [$^{13}C_6$] benzene, [^{13}C]-α-toluene and benzylsuccinic acid were obtained from Sigma-Aldrich (St. Louis, USA). [$^{13}C_6$] monochlorobenzene and [$^{13}C_7$] toluene were purchased from Chemotrade Leipzig (Germany). All the isotopically labeled compounds had a chemical purity higher than 99 %.

4.3.2 Field Sites (Zeitz, Bitterfeld)

Zeitz (Saxony-Anhalt, Germany). The benzene, toluene, ethylbenzene and o,m,p- xylene (BTEX) contaminated aquifer is located in the area of a former hydrogenation plant close to the town of Zeitz in Saxony-Anhalt, Germany. A detailed description of site hydrogeology and hydrochemical conditions of the contamination plume at the SAFIRA (Remediation research in regional contaminated aquifers) site was given in previous studies (Fischer et al., 2004; Vieth et al., 2005). The thickness of the upper aquifer varies between 4 to 6 m. Depth discrete investigation of geochemical and isotope parameters in Zeitz have been previously described (Stelzer et al., 2006a). In the source area BTEX exceeded concentrations of 900 mg L^{-1} and benzene and toluene were present in concentrations up to 850 mg L^{-1} and 50 mg L^{-1}, respectively. Ethylbenzene and xylenes are typically present in concentrations lower than 3 mg L^{-1}. The groundwater was sampled from the wells indicated in Fig. 4-2. At the test site the predominant electron acceptor used for biodegradation was sulfate (Vieth et al., 2005) and the contribution of methanogenic processes in the microbial transformation in the BTEX-plume is of minor importance (Fischer et al., 2004). Other electron acceptors such as oxygen, nitrate and iron play a minor role for the overall biodegradation processes at the site.

Bitterfeld (Saxony-Anhalt, Germany). The monochlorobenzene (MCB) contaminated aquifer is located near the town of Bitterfeld in Saxony-Anhalt, Germany. Concentrations up to 30 mg L^{-1} of MCB and lower concentrations of chlorinated aliphatic compounds (< 5 mg L^{-1}), benzene (< 0.77 mg L^{-1}), and dichlorobenzenes (< 3 mg L^{-1}) were found in the aquifer (Weiss et al., 2002). Due to the 150-year history of the chemical industry at the site, the time of spillage is not exactly known but chlorobenzenes have been produced at the industrial site since the first half of the last century. The hydrogeological situation is characterized by a shallow Quaternary aquifer system consisting of glaciofluvial sand and gravel and a deeper Tertiary aquifer system of micaceous sand overlying glauconite sand. A lignite layer overlaid by a Miocene clay layer formerly separated the Quaternary and Tertiary aquifer systems, which has been partially removed by open-cast mining. Today the two aquifer systems are

hydraulically connected at several locations. A Rupelian clay layer is the major regional aquitard of the Tertiary aquifer system (Heidrich et al., 2004a; Heidrich et al., 2004b).

The MCB plume stretches down-gradient at the bottom of a Quaternary hydrogeological channel structure in a south-easterly direction (Kaschl et al., 2005). This Quaternary channel is located above the lignite layer, the local aquitard, separating the Quaternary from the underlying Tertiary aquifer. Oxygen concentrations in the aquifer were generally below 1 mg L^{-1}. Significant nitrate concentrations were only found in the upper layers of the aquifer indicating that nitrate reduction was not a relevant terminal electron acceptor (TEA). Some iron and manganese reduction was indicated by low Fe(II) and Mn(II) concentrations. Elevated sulfide concentrations associated with sulfate depletion indicated sulfate reduction and sulfate concentrations of up to 1.48 g L^{-1} suggested that sulfate was the principle TEA in this aquifer (Kaschl et al., 2005). Methane was not found in the aquifer indicating that methanogenesis was a very unlikely electron acceptor process. The groundwater in Bitterfeld was sampled in a cross section A-B between 17 and 21 m below ground (Left fringe: wells SAF11, SAF12, SAF27, SAF28, and right fringe: SAF7, SAF30, SAF31, SAF23, see Fig. 4-7 and Fig. 4-8) of the lower aquifer in monitoring wells which are perpendicular to the contamination plume as described in details elsewhere (Kaschl et al., 2005). In addition, in well SAF 4/97 the upper, Quaternary aquifer was investigated during a depth discrete multilevel sampling campaign.

4.3.3 Isotope fractionation and calculations

The carbon isotope composition of contaminants and fatty acids is reported in δ-notation (‰) relative to the Vienna-PeeDee-Belemnite-Standard (V-PDB) with $^{13}C/^{12}C = (11237.2 \pm 2.9) \times 10^{-6}$ (Craig, 1957) (Eq. 4-1)

$$\delta^{13}C\,[‰] = \left(\frac{(^{13}C/^{12}C)_{Probe}}{(^{13}C/^{12}C)_{Standard}} - 1 \right) \times 1000 \qquad \text{(Eq. 4-1)}$$

The determination of the fractionation of stable isotopes caused by primary enzymatic transformation reaction of a compound during microbial degradation can be described with the Rayleigh equation (Hoefs, 1997; Mariotti et al., 1981). The Rayleigh equation (Eq. 4-2) can be used to relate the change in concentration (C_t/C_0) to the instantaneous change in isotope composition (R_t/R_0) by the isotope fractionation factor (α).

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0} \right)^{\left(\frac{1}{\alpha}-1\right)} \qquad \text{(Eq. 4-2)}$$

R_0 and R_t are the isotope ratios at the beginning of the transformation reaction (t = 0) and after a given time (t), respectively. C_0 and C_t are the corresponding concentrations at the beginning of the reaction (t = 0) and after a distinct time (t), respectively. The isotope fractionation factor α quantifies the extent of isotope fractionation. The isotope fractionation is often reported as the enrichment factor ε [‰] = (α -1) x 1000.

Isotope fractionation factors are determined in laboratory reference experiments with microcosms, column experiments or pure cultures where microbial degradation is the only sink of contaminants. In this case the change in isotope ratio (R_t/R_0) can be related unequivocally to the change in concentration (C_t/C_0). In a double logarithmic plot of C_t/C_0 and R_t/R_0, the isotope fractionation factor can be determined from the slope of the linear regression curve. Isotope fractionation factors (αD, αC, αCl) published until 2004 can be obtained from recent reviews (Meckenstock et al., 2004a; Schmidt et al., 2004) or can be obtained from a frequently updated data base (www.isodetect.de).

The isotope fractionation concept for quantifying the microbial *in situ* degradation is based on the fact that *in situ* biodegradation is the major process altering the isotope composition of priority contaminants such as BTEX, chlorobenzenes and chlorinated ethenes in ground water to a significant extent. Other processes such as sorption and volatilization may affect the isotope composition but do not alter the isotope composition to a significant extent in comparison to biodegradation. Therefore, the isotope composition of contaminants in ground water provides information about the cumulated extent of intrinsic transformation. The isotope fractionation caused by biodegradation leads to an enrichment of heavy isotopomeres in the residual fraction of the contaminant. A modified Rayleigh equation is used to quantify the microbial *in situ* degradation degree (Biodegradation; B[%]). B[%] represents the concentration decrease expected along a theoretical streamline plug flow without mixing and a single degradation process with a constant isotope fractionation factor. The change in concentration (C_t/C_0) down gradient from the source of contaminants is calculated employing the changes in the isotope ratio between the source (R_0) and a monitoring well (R_t) and an appropriate isotope fractionation factor (α) (Eq. 4-3).

(Eq. 4-2)
$$B[\%] = \left(1 - \frac{C_t}{C_0}\right) * 100 = \left[1 - \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\frac{1}{\alpha} - 1}\right)}\right] * 100$$

4.3.4 Preparation and incubation of *in situ* microcosms

The *in situ* microcosm experiments were performed using the BACTRAP system described elsewhere (Büning et al., 2005; Geyer et al., 2005). Briefly, Bio-Sep® beads (provided by K. Sublette, University of Tulsa, Tulsa, USA) were loaded with contaminants as carbon source

for microorganisms. The spherical beads, 2 to 3 mm in diameter, consist of powdered activated carbon (PAC) incorporated within an aramid polymer matrix (Nomex®). The beads have a porosity of 75%, an internal surface area greater than 600 $m^2\ g^{-1}$ and outer pores of 1-10 microns (Peacock et al., 2004; White et al., 2003). The beads were heated at 300 °C for 4 hours to remove organic residues and then 0.5 g of beads per trap were filled into perforated Teflon® tubes and fixed with glass wool plugs at both ends. The filled microcosms were autoclaved at 121°C for sterilization and hydration of the bead material and then air-dried for 24 hours. The microcosms were loaded with the substrates (benzene, toluene or chlorobenzene) via gas phase under reduced pressure. The substrate and the microcosms were placed in small glass containers and evacuated for at least 48 hours to 60 mbar in order to allow uniform adsorption of the contaminants on the beads. In order to keep the beads anoxic, the vacuum was released by filling oxygen free water into the glass container and the beads were stored under anoxic conditions until their deployment in the monitoring wells.

The BACTRAPs for the microcosm experiments in Zeitz were loaded with [$^{13}C_6$] benzene to a concentration of about 88 mg g^{-1} bead material and with [^{13}C]-α-toluene to 112 mg g^{-1}. The microcosms were deployed in the well Saf Zz 32/02 at a depth of 14 - 15 m (4 m below the groundwater table). The *in situ* microcosms for the experiments in Bitterfeld were amended with [$^{13}C_6$] chlorobenzene to a concentration of 38 mg g^{-1}. For control experiments, the BACTRAPs were loaded with non-labeled benzene, toluene or chlorobenzene in similar concentration. Material from these control microcosms was used for molecular biological studies and to investigate the isotope composition of fatty acids at natural abundance.

A multi level packer system (MLPS) was used for depth discrete sampling from well SAF 4/97 in Bitterfeld (Kaschl et al., 2005; Schirmer et al., 1995) which was equipped with a filter screen from 4.5 to 22.3 m depth below ground. The multi level packer system is a water-filled sock tube inserted into the well, which allows separating various sampling systems to be separated from each other at different depths. Small submersible pumps allowed depth-specific sampling without cross-currents. A detailed description of the multi-level sampling system is given by (Schirmer et al., 1995). The microcosms were deployed in this well for 49 d at a depth of 10.7 m below the surface. The well is located in the anoxic central part of the plume and by the conventional sampling 0.3 mg MCB L^{-1} was found. In the depth discrete sampling campaign, concentrations below 1.0 mg L^{-1} were found up to the depth of 18 m and the concentration increase to more than 13 mg L^{-1} in the deeper part of the well.

4.3.5 Groundwater sampling and analyses

Groundwater sampling for the determination of concentrations and isotope signatures was performed according to good sampling practice. No specific efforts are needed and the samples can be taken in parallel during conventional groundwater monitoring campaigns.

Sampling techniques are described in the literature for example according to the German Industrial Norm (DIN, 1985; DIN, 1996). The biological activity in the samples can be stabilized by adding $HgCl_2$, sodium azide or other stabilizers depending on the target compound. Water samples with target compounds resistant to hydrolysis such as hydrocarbons can be stabilized by rising or lowering the pH. For the sampling of BTEX and monochlorobenzene (MCB) a 1 L bottle was completely filled with groundwater. NaOH pellets were added in order to raise the pH to at least 10 and the bottle was closed with a Teflon lined screw cap.

The sensitivity of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) systems is lower than conventional gas chromatography-mass spectrometry (GC-MS) techniques and therefore GC-C-IRMS requires more analyte in order to obtain reliable data. For reproducible carbon isotope analysis about 12 ng carbon is necessary. For hydrogen isotope analysis 5 to 10 times more material is needed. Therefore, the efficiency of the extraction technique to enrich the target compound is critical for the lowest concentration that can be analyzed. Depending on the target, solvent extraction, solid phase micro extraction and purge & trap techniques have been shown to be appropriate for isotope analysis (Meckenstock et al., 2004a; Schmidt et al., 2004).

The solvent extraction procedure which is described in more detail elsewhere (Kaschl et al., 2005; Richnow et al., 2003a; Vieth et al., 2005) was employed for the isotope analysis of BTEX and MCB in this work. Briefly, depending on the estimated concentrations, 1-2 ml n-pentane were used for extraction of 1 L of groundwater Although this extraction is not quantitative, no isotope effects were associated with this extraction method (Dempster et al., 1997) and MCB and BTEX compounds can be analysed to concentration as low as some µg L^{-1}.

The beads of the microcosms were extracted using a dichloromethane-methanol-water mixture as solvent modified according to Bligh and Dyer (1959). After phase separation the dichloromethane phase containing the fatty acids (FA) was evaporated to dryness and derivated using a trimethylchlorosilane (TMCS) methanol mixture (1:8; v:v) as reactant for 2 h at 70 °C to obtain fatty acid methyl esters (FAME) (Thiel et al., 2001). After evaporation to complete dryness the FAME fraction was dissolved in n-hexane for subsequent analysis by GC-MS and GC-C-IRMS.

For the determination of concentrations and the identification or structural characterization of FAME and benzylsuccinic acid methyl ester by GC-MS, a Hewlett Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent Technology, Palo Alto, USA) was used. The carboxylic acid fraction was separated on a BPX-5 column (30 m*0.32 mm*0.25 µm; SGE, Darmstadt, Germany) with a temperature program of 120°C initial temperature for 4 min, heat at 4°C min^{-1} to 250°C, heat at 20°C min^{-1} to 300°C, and

hold for 10 min. FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco International) and quantified relative to an external standard mix or an internal standard (Henicosanoic acid, C21:0). The fatty acids are designated in the form of A:BωC where A is the number of carbon atoms, B is the number of double bonds and C is the distance of the closest double bond from the aliphatic end of the molecule (unsaturation, ω-nomenclature). The prefix i (iso) and a (anteiso) refer to methyl branching. The benzylsuccinic acid methyl ester was characterized by co-injection and comparison with mass spectra obtained for the authentic reference compound which was derivated as described below.

The carbon isotope composition of the carboxylic acids fractions was analyzed using a GC-C-IRMS system. It consists of a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Bremen, Germany). The performance of the measurement has been described previously (Richnow et al., 2003a). A BPX-5 column (50 m*0.32 mm*0.5; SGE, Darmstadt, Germany) was used for chromatographic separation with helium as carrier gas at a flow rate of 1.5 mL min^{-1} and a temperature program with initial temperature of 60 °C for 2 min, heat at 20 °C min^{-1} to 120 °C, heat at 2 °C min^{-1} to 300 °C, and hold for 20 min (Miltner et al., 2004). The carbon isotope composition analysis of BTEX and MCB on the same system was described previously (Kaschl et al., 2005; Vieth et al., 2005).

The methylation of carboxylic acids for gas-chromatographic analyses introduces an additional carbon into the structure of the analyte which affects its isotopic composition. Therefore the isotope signature of fatty acids ($\delta^{13}C_{FA}$) was corrected for the isotope effect due to derivatization FAME with methanol as described previously (Abraham et al., 1998; Abrajano et al., 1994; Goodmann and Brenna, 1992).

Molecular biological analyses of the beads from the exposed microcosms were performed according to the following procedure: For each sample, a total of 50 beads were transferred into 15 ml Falcon tubes containing 12 ml of TES buffer (50 mM NaCl2, 10 mM Na2EDTA, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride [Tris HCl], pH 8.0) with 1% sodium dodecylsulfate (SDS). Five cycles of freeze-thaw lysis were performed, each with 5 min in liquid nitrogen (-196 °C) and 5 min at 65 °C in a shaking water bath, each cycle was interrupted by 10 s of vigorous vortexing. DNA was then extracted with phenol-chloroform, as described elsewhere (Schwieger and Tebbe, 1998; Schwieger and Tebbe, 2000). The isopropanol precipitated and purified DNA of each tube was resuspended in a total of 40 µl of 10 mM Tris in 10 µl aliquots. Aliquots were kept at 4 °C for further analysis or stored at -20 °C.

Partial 16S rRNA genes, including two variable regions, were amplified from total DNA by PCR, using primers Com1 and Com2ph (Schmalenberger et al., 2001). The PCR products were converted into DNA-single strands by lambda-exonuclease digestion, following the protocol of Schwieger and Tebbe (1998). Genetic profiles of each sample were generated by single strand conformation polymorphism (SSCP) on non-denaturing polyacrylamide gels, as described elsewhere (Dohrmann and Tebbe, 2004; Tebbe et al., 2001). Single bands were selected for further DNA-sequencing after PCR-amplification and cloning in Escherichia coli. Sequencing is described in the same references. Consensus sequences were compared to database sequences using the Fasta Nucleotide Database Query provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta).

4.4 Results and Discussion

4.4.1 Benzene and Toluene degradation at the SAFIRA reference site Zeitz

Stable isotope fractionation analyses (cumulated biodegradation approach)

In order to obtain information about the degradation processes that have already been performed by the microorganisms at a contaminated site, the cumulated biodegradation approach was developed based on stable isotope fractionation analysis (SIFA). The overall workflow is summarized in Fig. 4-1. First, the concentrations of BTEX in the wells of the SAFIRA reference site Zeitz were analyzed where benzene is the predominant compound (Fig. 4-2A). Next, the natural carbon isotope composition of benzene in the course of the plume was determined (Fig. 4-2B). The centre of the plume with concentrations up to more than 950 mg L^{-1} was characterized by an average isotope composition of -28.5 ± 0.5‰. Down gradient the concentration decreases to below 1 mg L^{-1} at the fringes of the plume and the benzene isotope composition was enriched to maximum values of -24.4 ‰ indicating biodegradation (Fischer et al., 2004).

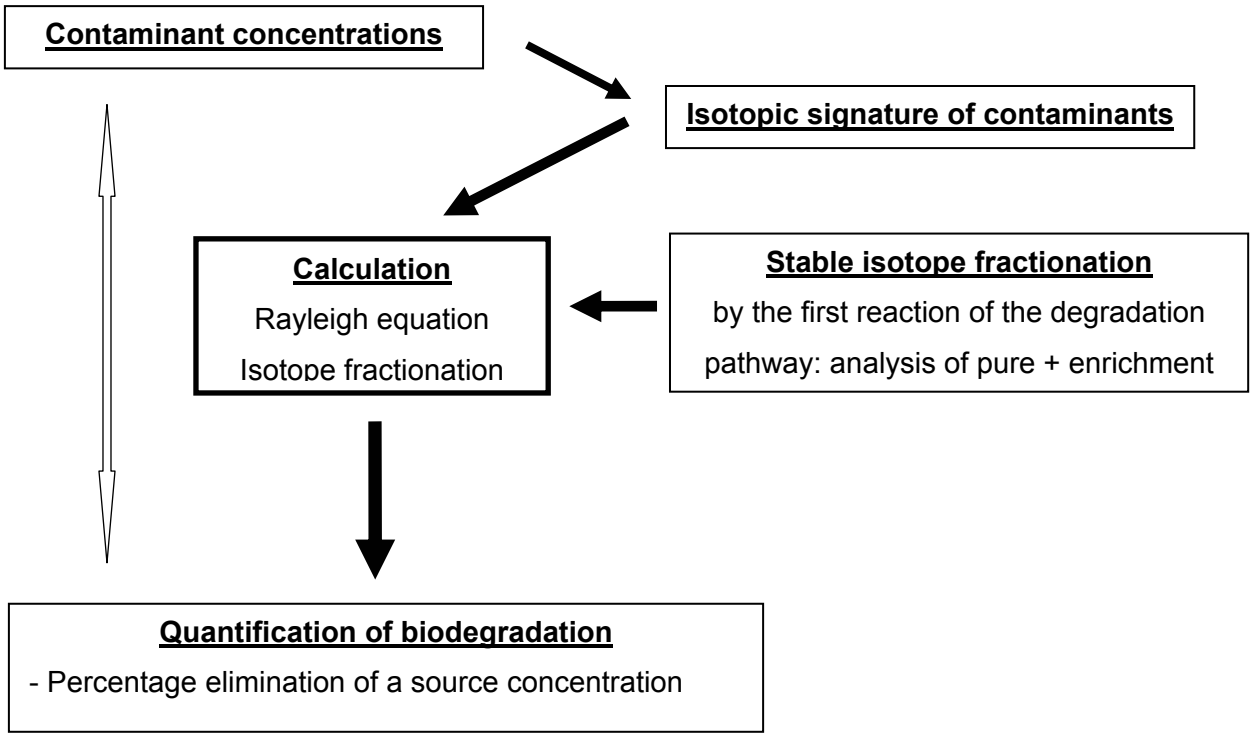


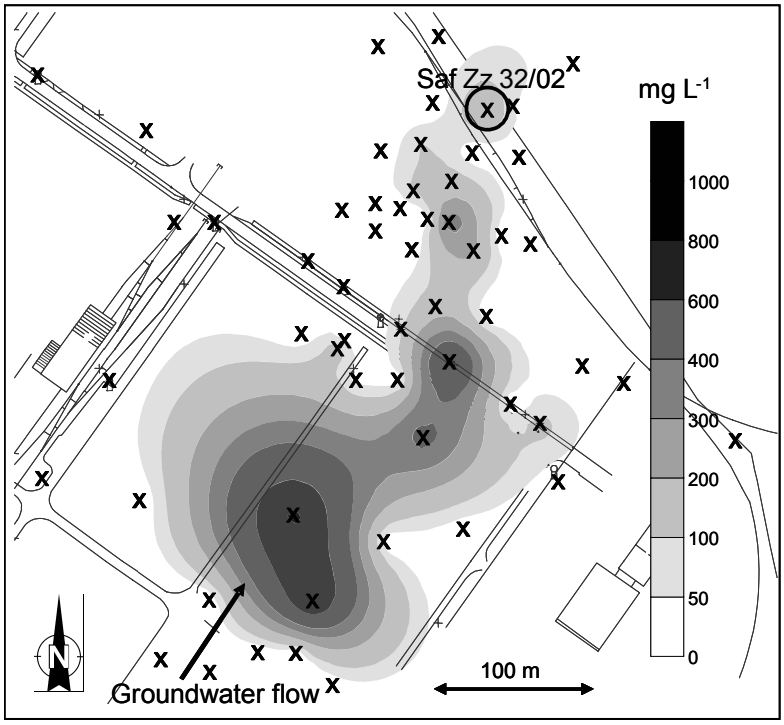
Fig. 4-1: Approach I: Assessment of the cumulated biodegradation in a contaminated aquifer by use of stable isotope fractionation analysis (SIFA) at natural abundance; for details see text.

In order to quantify the extent of biodegradation, it is necessary to determine how a given microbial metabolic pathway fractionates $^{13}C/^{12}C$ during transformation. Isotope fractionation by microbial degradation is a result of the higher transformation rate of lighter isotopomers compared to heavier isotopomers by enzymatic reactions (Bisswanger, 2000; Galimov, 1985). This kinetic isotope fractionation enriches the amount of the ^{13}C isotopomer in the residual substrate. The isotope fractionation factor (α) describes the relation between the decrease in concentration and change in the isotope ratio during the degradation reaction and can be described by the Rayleigh equation (Eq. 4-2). The isotope fractionation factor can be obtained in reference degradation experiments with pure cultures or at least well defined cultures in which the degradation pathway or the redox conditions are known and in which no other elimination process may alter the concentrations except biodegradation (Meckenstock et al., 2004a). The extent of isotope fractionation depends on the initial enzymatic degradation reaction of the respective biochemical pathway. Without the knowledge about terminal electron acceptor process and degradation pathway, isotope fractionation factors from microcosm studies are not very precise because they may vary upon experimental conditions. Finally, with an appropriate isotope fractionation factor, the percentage of biodegradation along a flow path in a contamination plume can be calculated by the data from the SIFA using equation 4-3.

The metabolic degradation pathway governs the extent of isotope fractionation and therefore, it is important to apply the appropriate isotope fractionation factor for calculation. For

example, from pure cultures able to degrade toluene it is known that the aerobic degradation and the resulting isotope fractionation can vary significantly depending on the initial oxidation reaction at the methyl group or at the aromatic ring system (Morasch et al., 2002). However, under anaerobic conditions the benzylsuccinate pathway attacking toluene at the methyl group shows quite similar isotope fractionation factors under nitrate- iron, and sulfate reducing conditions (Heider et al., 1999; Meckenstock et al., 1999; Morasch et al., 2001; Spormann and Widdel, 2000; Widdel and Rabus, 2001). At a contaminated site, the microbial pathway relevant for the degradation can be deduced by the redox conditions in the aquifer or by assessment of the primary metabolites of the compound of interest (Griebler et al., 2004b). In case of toluene, benzylsuccinate was detected in Zeitz, which supports the assumption that sulfate reducing conditions govern the anaerobic degradation of toluene in the aquifer. Similarly, the fractionation of benzene also varies under these conditions (α = 1.0036) but is lower under methanogenic conditions (α = 1.002) (Mancini et al., 2003). Because sulfate was the major electron acceptor in Zeitz, here we used the fractionation factor for benzene degradation under sulfate reducing conditions for calculating the *in situ* degradation. To show the uncertainty, we also calculated the degradation with a fractionation factor characterizing methanogenic conditions (α = 1.002). Using the larger fractionation factor (α =1.0036) characteristic for sulfate reducing conditions, the interpretation of the benzene isotope composition give a more conservative estimate (up to 69 % degradation) whereas the calculation with the lower factor (α = 1.002) indicative for methanogenic conditions suggest a higher degradation (up to 88%). A field survey over the contamination plume enabled mapping of degradation of contaminants (Fig. 4-3). A previously published monitoring campaign in Zeitz show similar results (Vieth et al., 2005). Examinations of other wells or other BTEX contaminated sites are described elsewhere (Büning et al., 2005; Geyer et al., 2005; Stelzer et al., 2006a; Stelzer et al., 2006b).

A



B

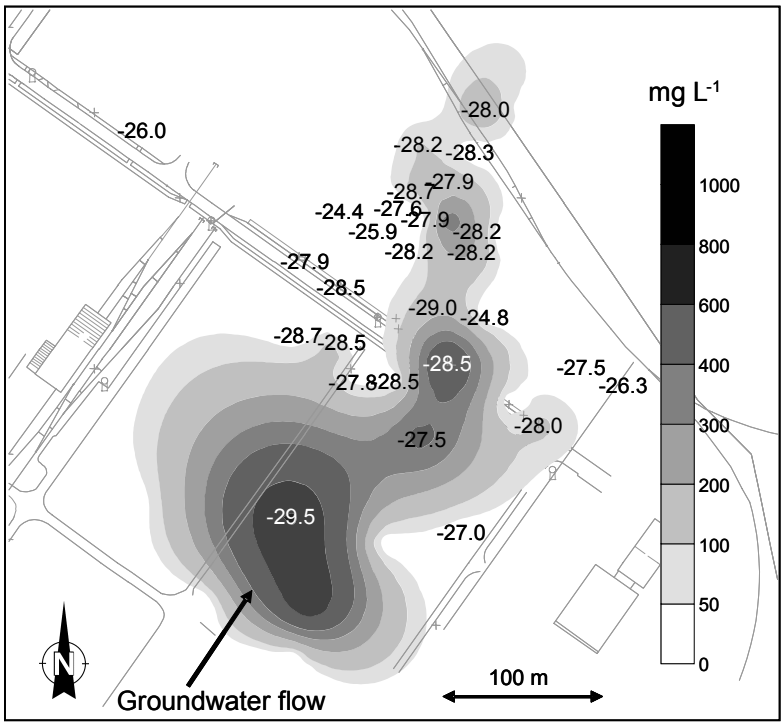


Fig. 4-2: A) Spatial distribution of the benzene contaminations in the wells of the Zeitz aquifer in 2002 and 2004 (X = location of sampling wells; Saf Zz 32/02 well of the *in situ* microcosm experiment). B) Spatial distribution of the carbon isotope composition of benzene in the wells of the Zeitz aquifer.

In addition, a number of field studies exist where the enrichment of heavy isotopes in the residual fraction of mostly BTEX or chlorinated ethenes was used to estimate the *in situ* degradation (Chartrand et al., 2005; Fischer et al., 2004; Griebler et al., 2004b; Lollar et al.,

2001; Meckenstock et al., 2002; Morrill et al., 2005; Peter et al., 2004; Richnow et al., 2003a; Richnow and Meckenstock, 1999b; Richnow et al., 2003b; Steinbach et al., 2004; Vieth et al., 2001; Vieth et al., 2005; Vieth et al., 2003). For other compounds such as naphthalenes, chlorinated benzenes, phenols, fuel oxygenates (MTBE, ETBE, (Zwank et al., 2005)) less experience exists showing the need of further field studies. The rapid increase of the references within the last five years shows a broad acceptance of the general approach and the reliability of the technique will be further improved with increasing data sets for isotope fractionation factors. At present a range of fractionation factors for several compounds under different environmental conditions is available by recent reviews (Meckenstock et al., 2004b; Schmidt et al., 2004) and data bases (www.isodetect.de). Particularly for the mono-aromatic BTEX contaminants, the applicability of the technique is highly reliable. However, at present the cumulated biodegradation or SIFA approach is of limited value for contaminants such as tert-butyl alcohol, tri-, dichloroethylene, and vinylchloride which can be both, substrate or metabolite. Aquifers contaminated by tetra- and trichloroethene may show a sequential transformation pattern with varying isotope compositions which are not easy to interpret (Martin and Ertl, 2005).

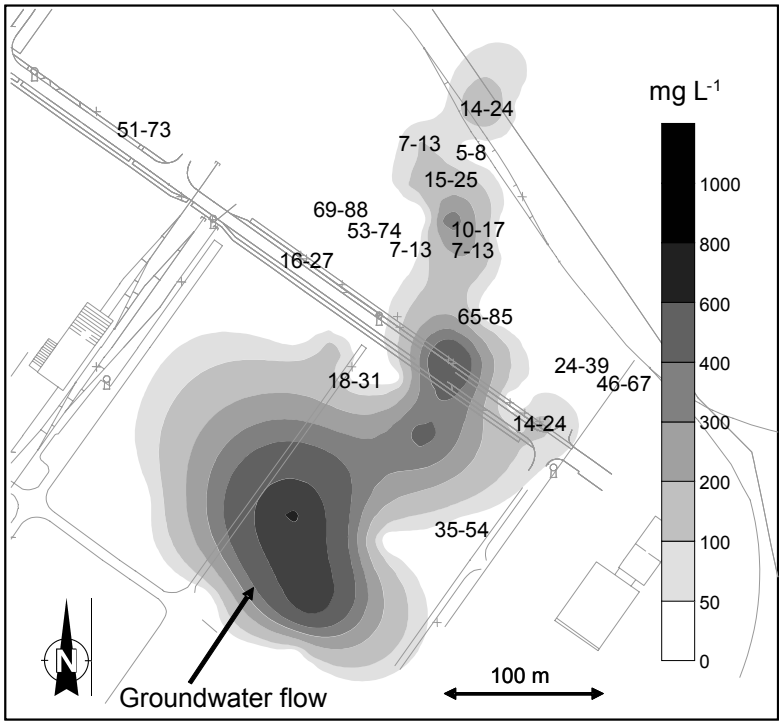


Fig. 4-3: Percentage of benzene degradation in the wells of the contamination plume calculated by use of equation 3 (isotopic composition of the source [R_0] = -28.5‰, α = 1.0020 (methanogenic) – 1.0036 (sulfate reducing); modified from (Fischer et al., 2004).

In situ microcosms with stable isotope labeled substrates (BACTRAP approach)

At many sites, however, there is only limited access to the contamination plume due to the small number of wells or the insufficient data base for the respective compounds. Therefore, a method to prove the microbial degradation of the contaminants is necessary which can be

applied directly in the groundwater monitoring wells. In order to provide this proof and to estimate the turnover and the relevant pathway, the *in situ* microcosm approach was developed (Geyer et al., 2005). The system consists of beads containing activated carbon in a granular shape that are loaded with ^{13}C-labeled contaminants as shown for benzene or toluene at the Zeitz site. The beads provide a large amount of internal surface area for colonization or attached growth of degrading bacteria (biofilm formation). If the indigenous bacteria of the well colonize the beads and productively consume the contaminants under *in situ* conditions, the ^{13}C-labeled carbon will be incorporated into the growing cells and can be traced within biomarker molecules such as fatty acids or nucleic acids. The overall workflow of the approach is presented in Fig. 4-4.

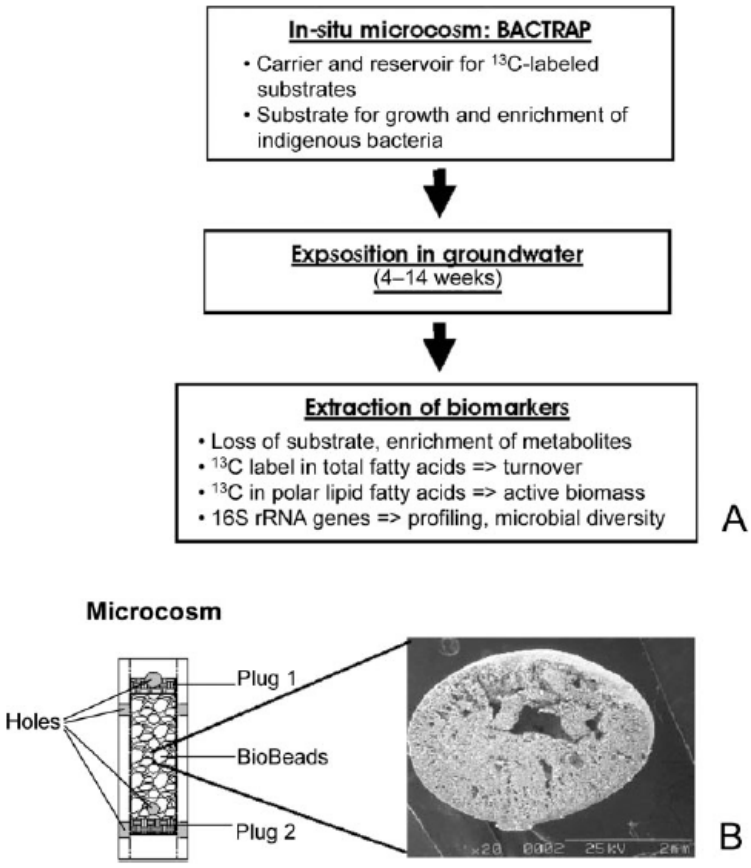


Fig. 4-4: Approach II (A): Assessment of biodegradation potentials in a contaminated aquifer by use of *in situ* microcosms (BACTRAPs) with tracer compounds labeled with stable isotopes; (B): microcosm containing Bio-Sep® beads. (Picture: Copyright (2003) from Environmental Forensics, 2003, 4(1) by White et al.; reproduced by permission of Taylor & Francis Group, LLC., http://www.taylorandfrancis.com)

In order to analyze the microbial activity, sterilized *in situ* microcosms were loaded with ^{13}C-labeled benzene or toluene and were exposed for 32 d in well Saf Zz 32/02 in Zeitz. Non-amended microcosms served as controls. After exposure and development of the biofilms on the beads, the residual substrates and the lipid biomarkers were extracted and analyzed by means of mass spectrometry. About 88 to 112 mg of benzene and toluene per g of bead

material in the microcosm was initially applied and around 16 mg g^{-1} was recovered after exposure (Tab. 4-1). Isotopic analysis of the residual amounts of ^{13}C-benzene or ^{13}C-toluene in the recovered microcosms from Saf Zz 32/02 in September 2003 showed only a slight accumulation of the contaminants from the aquifer in the bead material in this well. In July-August 2004, however, an accumulation of up to 5.6 mg g^{-1} from the aquifer was observed in the controls after 112 d of exposure as a result of the much higher contaminant concentrations of about 300 mg L^{-1} at this time. The overall standard deviation of the recovered substrates from the BACTRAP systems amounted to < 10 %.

Tab. 4-1: Concentrations and ^{13}C abundance of contaminants in the *in situ* microcosms (BACTRAP®s) before and after exposure in well Saf Zz 32/02 (modified from Geyer et al., 2005).

	Before exposure		After 32 d		
	^{13}C (atom%)	load (mg g^{-1} a))	^{13}C (atom%)	conc. (mg g^{-1} a))	loss (%)
benzene	0.1	88 ± 1.0	0.1	15.8 ± 0.2	82
toluene	0.1	112 ± 1.1	0.1	16.0 ± 1.4	85
[$^{13}C_6$]-benzene	98.0	88 ± 0.2	98.0	15.8 ± 0.2	82
[$^{13}C_1$]-α-toluene	14.0	112 ± 1.2	14.0	17.8 ± 1.4	84
control	-	0.0	0.1	0.46 (benzene)[b)]	-
(control)	-	0.0	0.1	5.6 (")[c)]	-

a) per g bead material

b) non-loaded control; accumulation from aquifer contaminant plume in September 2003

c) non-loaded control; accumulation from the aquifer after 112 d of exposure in July-August 2005 showing much higher benzene concentrations and lower biofilm formation

Total lipid fatty acid (TLFA) patterns of extracts and their isotopic compositions, as well as polar lipid fatty acids (PLFA) of the beads from the microcosms exposed in well Saf Zz 32/02 were analyzed and the TLFA patterns are shown in Fig. 4-5. TLFA from both, ^{13}C-benzene and ^{13}C-toluene amended microcosms showed ^{13}C-enrichment of up to δ = 13360 ‰ in the fatty acids, providing clear evidence for benzene and toluene biodegradation with transformation of the ^{13}C into the microbial biomass. The decrease in contaminant concentrations of the microcosms can thus be considered to be attributable to microbial activity. Extracts from ^{13}C-toluene-amended beads showed incorporation of ^{13}C into more or less the same fatty acids as found in the ^{13}C-benzene microcosm.

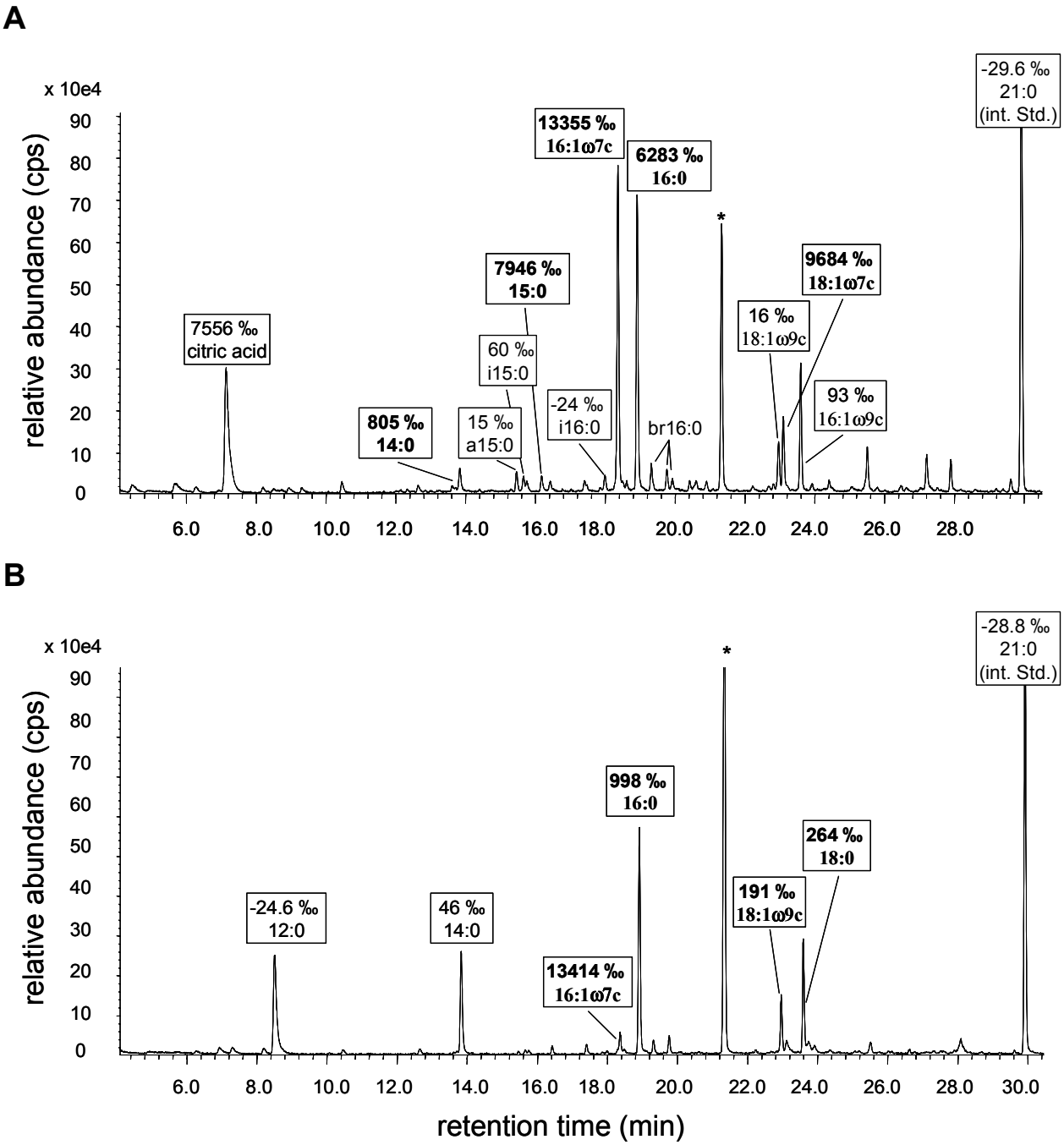


Fig. 4-5: GC-MS profiles and isotope enrichment of fatty acids in total lipid extracts obtained from the [$^{13}C_1$]-α-toluene (A) and [$^{13}C_6$] benzene (B) amended microcosms deployed in well Saf Zz 32/02 in the Zeitz aquifer for 32 days. Major fatty acids with retention time in brackets given in minutes were 14:0 (13.83), i15:0 (15.46), a15:0 (15.66), 15:0 (16.42), i16:0 (17.98), 16:1w7c (18.36), 16:0 (18.91), 18:1w9c (22.96), 18:1w7c (23.10), 18:0 (23.59), br16:0 (19.92, 20.41, 20.60) with undetermined position of methyl-branching, * phthalate (21.31) from extraction process. (The $\delta^{13}C$ values for the isotopic composition of the respective methyl esters represent the isotopic shift compared to the PDB standard.)

Estimation of the ^{13}C recovery in the biomass. The incorporation of ^{13}C into the fatty acid fraction causing a shift of up to δ 13360 ‰ indicates that [^{13}C]-α-toluene (~14.5 atom %, see Tab. 4-1) was more intensively incorporated into microbial biomass than [$^{13}C_6$] benzene (98 atom %). This was expected because toluene is considered to be more easily degradable

than benzene. For 16:1ω7c, the isotope composition was 13360 ‰ (13.9 atom %), very similar to the toluene substrate, which indicates that at least one organism producing this fatty acid used the applied toluene nearly exclusively as a carbon source. The isotope signatures of C15:0, C16:0 and 18:1w7c of 7946 ‰ (9.1 atom %); 6283 ‰ (7.6 atom %); and 9684 ‰ (10.7 atom %), respectively, demonstrate that other organisms in the consortium assimilated the labeled C from toluene to a major extent but not exclusively. Fatty acids with a lighter isotope composition such as for 18:0 with 92 ‰ (1.2 atom %) indicated that some organism colonized the *in situ* microcosms using other carbon sources than the labeled toluene, e. g. other contaminants or natural organic matter to a major extent.

The most intensively labeled fatty acid on the [$^{13}C_6$] benzene amended microcosms was 16:1ω7c with an isotope composition of 13410 ‰ corresponding to 13.9 atom %. The isotope composition of [$^{13}C_6$] benzene was about 98 atom % and therefore around 1/7th of the carbon was derived from the labeled benzene. Thus, the majority of carbon was derived from non labeled carbon sources which may comprise non-labeled benzene from the contamination, natural organic carbon sources or CO_2 used for synthesis of fatty acid. The C16:0 and C18:0 fatty acids were much lower labeled indicating that some organism use benzene only to a minor extent for biosynthesis. Almost non-labeled fatty acids (C12:0) suggest that parts of the microbial community colonizing the beads are not involved in biodegradation of benzene and use other carbon sources exclusively.

The concentrations and isotope compositions of individual fatty acids were used to calculate the amount of ^{13}C converted from the contaminant according to the method described by Richnow et al. (1999a). Since the isotope composition of fatty acids from the non-amended *in situ* microcosms were typically between -35 and -25 ‰, it was considered that an isotope composition enriched to more than -20 ‰ was significantly affected by the transformation of the labeled contaminants. On the benzene amended trap about 3.1 nmol ^{13}C g^{-1} bead material corresponding to 43 ng ^{13}C g^{-1} labeled benzene was bound in the fatty acid fraction. The ^{13}C-α-toluene derived carbon bound within fatty acids was an order of magnitude higher. The amount of 456 nmol toluene derived carbon g^{-1} corresponds to the equivalent of 6062 ng labeled toluene. Therefore, the assimilation of C from toluene was more than 140 times higher than that of benzene. Considering that fatty acids account for up to 5 % of the total biomass carbon and that the carbon isotope signature in lipids is similar to that in other fractions of the biomass, the overall amount of labeled carbon bound within the biomass is 20 times higher than found in the fatty acids. If we expect furthermore, that around 95% of the carbon source is metabolized and 5 % is used for biosynthesis of biomass ($y_{x/s}$ = 0.005), the total productive metabolisation can be estimated as 2400 µg toluene and 17.2 µg benzene during incubation in the aquifer. These values only account for 2.5 % of the toluene

loss and 0.024 % of the benzene loss. The ^{13}C-label of benzene, however, was highly diluted due to the background concentrations of benzene from the aquifer.

Phospholipid fatty acids (PLFA) are parameters for microbial membrane phosphoglycerolipid content and thus an indicator for viable cells (Guckert et al., 1985). The total PLFA amounts were highest in the ^{13}C-toluene microcosms with 8870 pmol and 8480 pmol in the ^{12}C-toluene microcosm (Geyer et al., 2005). The microcosms with ^{13}C- or ^{12}C-benzene and the control contained 5860 pmol, 2410 pmol and 860 pmol PLFA, respectively. Applying the conversion factor of 1.4 - 4.0 × 10^4 cells pmol^{-1} of PLFA, these values correspond to 2.3 x 10^7 - 2.4 x 10^8 bacterial cells with the size of *Escherichia coli* (White et al., 1996) verifying a considerable microbial colonization in the bead material of the microcosms.

Even though no strictly anaerobic pure cultures able to degrade benzene under iron or sulfate reducing conditions are available yet, the *in situ* microcosm approach with ^{13}C-labeled compounds could prove the microbial *in situ* degradation of benzene under strict anaerobic conditions in Zeitz. At present, only nitrate or perchlorate reducing pure cultures responsible for the degradation of benzene have been described (Chakraborty and Coates, 2005). The transformation of ^{13}C-carbon from the labeled substrate into microbial fatty acids provided clear evidence for the assimilation of the pollutant resulting in the formation of biomass. This easy to handle and cost-effective microcosm approach can be used to provide the information required to implement Monitored Natural Attenuation (MNA) as requested by the OSWER directive (US-EPA, 1999) for approval of Natural Attenuation as a treatment measure for contaminated sites.

Metabolites. The activated carbon of the beads may enrich degradation metabolites that can be used to identify the involved degradation pathways. The formation of labeled benzylsuccinate upon toluene degradation was observed in the Zeitz aquifer (data not shown) and is indicative for the anaerobic degradation pathways of aromatic compounds in microorganisms using sulfate and iron as terminal electron acceptors (Heider et al., 1999; Spormann and Widdel, 2000; Widdel and Rabus, 2001). The non-labeled analogue was also found in the control microcosms indicating a degradation of the aquifer contaminants under the respective conditions. Reusser et al. (2002) applied push and pull experiments with deuterium labeled toluene and xylene to verify *in situ* degradation of the compounds in a contaminated aquifer. Although deuterium labeled contaminants may be useful tracer compounds for proving *in situ* degradation, much higher amounts of labeled substances are needed during push and pull experiments compared to the BACTRAP approach. The application of *in situ* microcosms as described here can significantly reduce the amount of labeled material and gives in principle, information similar to that obtained from push and pull experiments. However, by using ^{13}C-labeled compounds in the *in situ* microcosms the

transformation into biomass can be traced by analyzing the biomarker molecules providing the proof of principle for biodegradation.

Biodiversity. The biodiversity of the microbial biomass from *in situ* microcosms exposed in well Saf Zz 32/00 in summer 2004 over a period of 7 to 98 d was characterized by cultivation independent molecular biological approaches based on PCR-amplified partial 16S rRNA genes from total DNA (Scow and Hicks, 2005). Total DNA was directly extracted from the *in situ* microcosms and the PCR products amplified from the various samples were compared to each other on the basis of their SSCP profiles (Fig. 4-6). The community profiles were typically composed of approx. 20 bands with about 5 to 10 bands being more dominant. Most of the profiles generated from *in situ* microcosms taken after different incubation times were relatively similar to each other, indicating a highly reproducible colonization pattern of the bead material. For most samples analyzed, the bacterial community compositions on the benzene and toluene loaded microcosms were not severely altered in comparison to the non-amended controls although highly labeled microbial fatty acids extracted from the ^{13}C-experiments showed a substrate degradation and incorporation into biomass (Tab. 4-1). This indicates for a coordinated degradation by certain members of the indigenous microbial community colonizing the *in situ* microcosms and channeling of the ^{13}C carbon into the microbial food web. However, the isotope composition showed that these organisms also used other carbon sources. Benzene and toluene were the major carbon sources but other BTEX compounds, contaminants, or humic substances were certainly present and may be used as additional carbon sources.

The comparison of the profiles of the non-amended controls with the loaded microcosms showed that the DNA and FA patterns were only slightly different. This reflects the fact that the bead material (activated carbon) of the control (blank) extracted a certain amount of contaminants in particular benzene from the contaminated aquifer (Tab. 4-1) leading to similar conditions in the non-loaded and benzene-loaded microcosms. A comparison of the SSCP-profiles of the bead samples with the profiles of the groundwater samples from the same well showed that with the exception of two bands all bands found in the water were also present on the beads; conversely, only three bands found on the beads were not present in the groundwater samples (Fig. 4-6A). Hence, it can be concluded that the bead material provided a nearly non-selective surface for the growth of the indigenous microbial community from BTEX contaminated groundwater.

DNA-patterns from the toluene loaded microcosms were also highly similar indicating that toluene degraders were already members of the colonizing community and may have conducted the toluene degradation at the site. Cluster analysis of the profiles (Fig. 4-6A) revealed four major groups (Fig. 4-6B). Cluster I contained communities from all microcosms

collected after short time exposure (24 d) or without loaded substrates (T1, see also legend of Fig. 4-6).

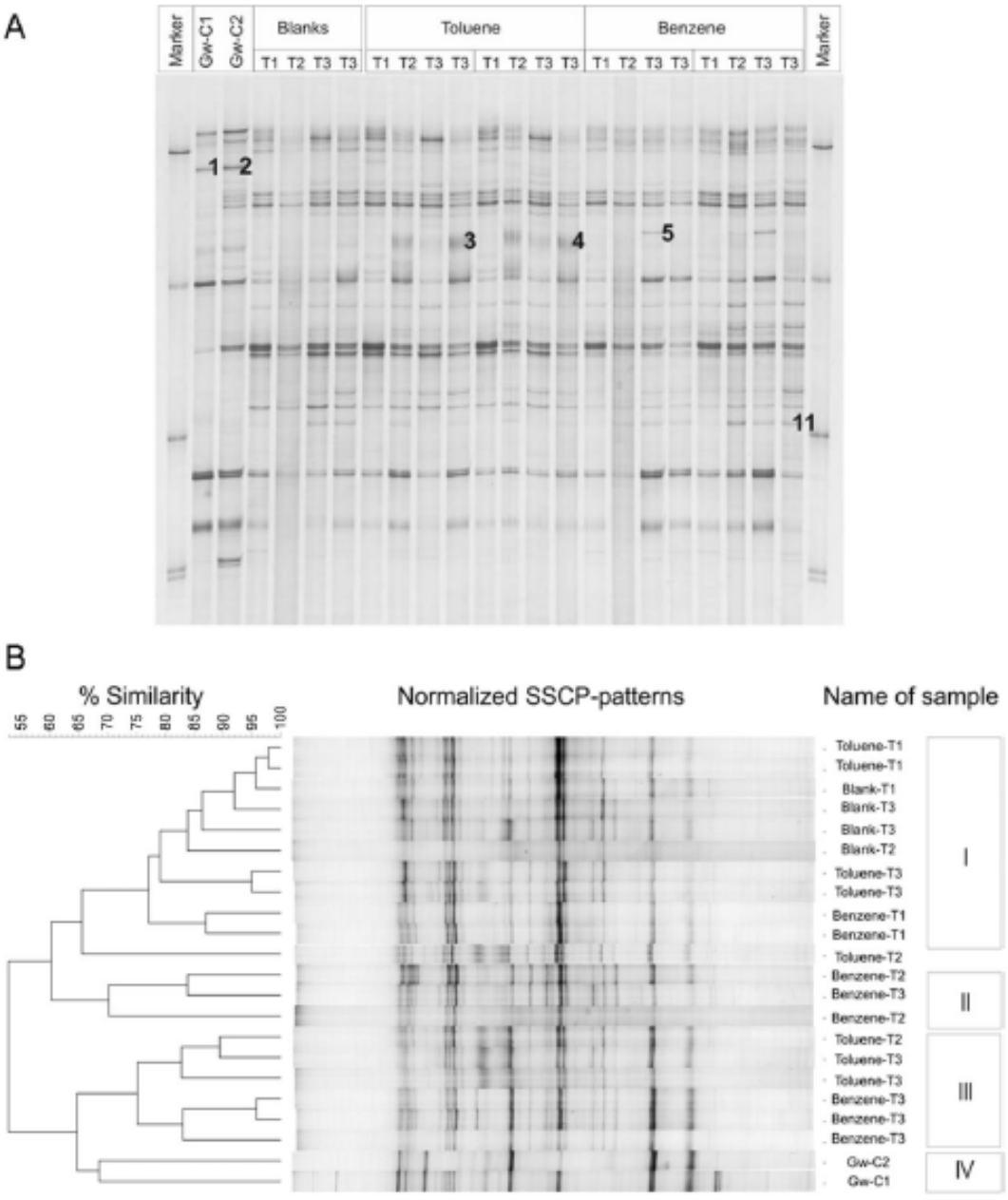


Fig. 4-6: SSCP Gel (A) and digital image analyses of SSCP-profiles (B) of PCR amplified 16S rDNA isolated from groundwater and microcosms loaded with toluene or benzene and controls (blanks) after exposure for 24 d (T1), 49 d (T2), and 92 d (T3) in well Saf Zz 32/02. Selected bands of these profiles were sequenced for identification, numbers indicated in the gel image can be found in Tab. 4-2.

In addition, two toluene loaded microcosms from a later sampling (T3) were found in this group. Cluster II contained samples from microcosms loaded with benzene. Other replicates of this treatment and sampling were found in Cluster III which mainly contained samples from substrate loaded treatments, collected later during the kinetic study. As expected on the one hand, the community profiles generated from the surrounding groundwater was different from the biofilm material extracted from the beads (Cluster IV), but on the other hand, similarities

between both sample types were evident. This indicated that in fact the dominant bacteria from the surrounding groundwater contributed to the biofilm communities. Interestingly, the similarity of the groundwater samples was higher with Cluster III (later samplings) than that with Cluster I, which can be explained by the fact that several of the bacteria from the aquifer were only slowly growing on the bead material.

Due to the similarity of the profiles to each other it was in most cases not really possible to identify bands which were highly characteristic for a certain substrate or period of incubation. However, for toluene, a characteristic band seems to be generated during the incubation (Fig. 4-6, band 3 and 4). DNA-sequencing of selected bands revealed that most bacteria in this community were Proteobacteria from the Alpha- und Beta-subgroup (Tab. 4-2). Similarities to known sequences in the database for these partial rRNA genes ranged from 85 to 100 %. Complete similarity was found with an uncultured bacterial clone isolated from monochlorobenzene contaminated groundwater (Alfreider et al., 2003). Other sequences, which were derived from the toluene-specific bands, were found to originate from *Azoarcus* sp., one of them most closely related to an organism that has been shown to degrade toluene and *m*-xylene under denitrifying conditions (Barragan et al., 2004; Zhou et al., 1995). Despite the fact that 16S rRNA genes are generally not reliable indicators for bacterial functions, the so far characterized sequences give no rise to assume growth of sulfate-reducing or other strictly anaerobic bacteria but rather indicates the presence of bacteria capable of growing under denitrifying conditions. Other sequences indicated α-proteobacteria adapted to the exposition with metals, assuming physiological similarities with their closest relatives (Lopez-Archilla et al., 2004; Stein et al., 2002). However, it is known from other strictly anaerobic bacteria e. g. *Dehalococcoides* sp. that no PCR products were found with the universal COM1/COM2 primers.

Tab. 4-2: Characterization of rRNA genes isolated from groundwater and microcosms loaded with toluene or benzene and controls after exposure in well Saf Zz 32/02 (see also Fig. 4-6)

Band No. [1)]	Length [bp]	Simi-larity [%]	Closest relative or sequence (Accession No.)	Phylogenetic group	Source of isolation or habitat of closest relative or relevant metabolic acitivity of closest relative
1	370	100	uncultured bacterium (AY05094)	Betaproteobacteria	monochlorobenzene contaminated aquifer
2	205	85	*Acidovorax* sp. (AJ864847)	Betaproteobacteria	high mountain lake habitat
3	370	99	*Azoarcus* sp. (AF515816)	Betaproteobacteria	degradation of toluene and benzene under denitrifying conditions
4	360	95	*Azoarcus denitrificans* (L33690)	Betaproteobacteria	growth on toluene under denitrifying conditions
5	350	93	uncultured bacterium (AY475201)	Alphaproteobacteria	from metal rich, acidic river Tinto (Spain)
11	210	90	uncultured bacterium (AF418953)	Alphaproteobacteria	from metal rich freshwater reservoir

[1)] PCR-products obtained from bands indicated in figure 6A; no products were obtained from other bands.

4.4.2 Monochlorobenzene degradation at the SAFIRA reference site Bitterfeld

Stable isotope fractionation analysis (cumulated biodegradation approach)

Monochlorobenzene (MCB) is a xenobiotic compound which has been commonly used for chemical manufacturing processes over decades and is a common groundwater contaminant at many chemical production sites in Europe and the United States (Arneth et al., 1989; Schiedeck et al., 1997). In addition, higher chlorinated benzenes can be degraded via reductive dechlorination to MCB under anaerobic conditions (for review see Van Agteren et al., 1998) and may be also produced from the microbial transformation of hexachlorocyclohexane (HCH) in anoxic aquifers (Phillips et al., 2005; Van Eekert et al., 1998). MCB is the major contaminant of the pollution plume in the Quaternary anoxic aquifer in Bitterfeld. The aquifer belongs to the regional aquifer system, in which about 200 million m^3 of groundwater are contaminated with predominantly chlorinated compounds (Heidrich et al., 2004a; Heidrich et al., 2004b). Field studies demonstrating the anaerobic microbial transformation of MCB are completely lacking. Thus, no pathways and mechanisms of anaerobic transformation of MCB are yet known. Taking into account that the plume formed in the aquifer several decades ago, a significant selection pressure for MCB degrading microorganisms can be expected. Therefore, assuming biodegradation we applied the SIFA approach (Fig. 4-1) to the complex contamination plumes in Bitterfeld (Kaschl et al., 2005).

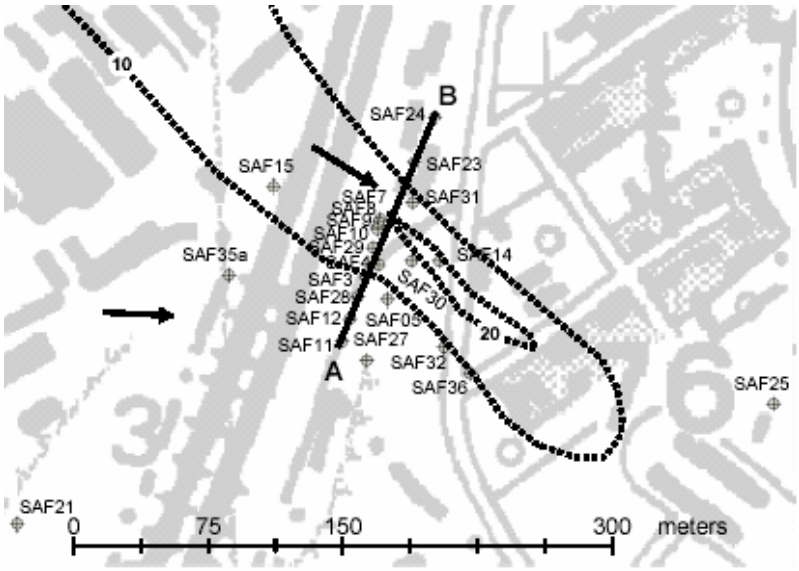


Fig. 4-7: Monitoring wells and distribution of the monochlorobenzene plume in Bitterfeld modified from (Kaschl et al., 2005). Concentrations are given by the dotted lines in mg L^{-1}. The arrows indicate the groundwater flow in November 2002. The solid line (A to B) represents the position of the cross-section perpendicular to the monochlorobenzene plume at the location of the SAFIRA research station. (Saf 4 = monitoring well for the vertical analysis).

The MCB plume stretches downgradient in a SE-direction, following a Quaternary hydrogeological channel structure oriented in a NW/SE direction, which strongly affects

contaminant flow. Since the access to the groundwater by wells was limited, we decided to analyze a cross section of wells A-B perpendicular to the groundwater flow in the lower aquifer which covers the concentration gradients at both sides of the plume (Fig. 4-7). The concentrations of MCB increased up to 20 mg L^{-1} in the centre of the plume and the isotopic signatures increased from values of -26.7 ‰ at the centre to -23.0 ‰ at the fringes of the plume (Fig. 4-8 left side) showing a significant ^{13}C-enrichment in the residual MCB at the fringes. In addition, we analyzed the vertical distribution of MCB and its isotope signature in the upper aquifer by multilevel sampling in well SAF 4/97 showing the same trends with a clear vertical MCB stratification (Fig. 4-8 right side). The ^{13}C-enrichment found at the fringes of the plume relative to the source area of about 4 δ units clearly shows the occurrence of *in situ* biodegradation of MCB. This would result in a field isotope fractionation factor of α = 1.0005. However, field fractionation factors are always lower than factors obtained from laboratory experiments in closed systems in which only biodegradation can diminish the concentrations. In the field, the concentrations may be affected by abiotic effects leading to much smaller fractionation factors suspending the calculation of the effective biodegradation according to equation 4-3.

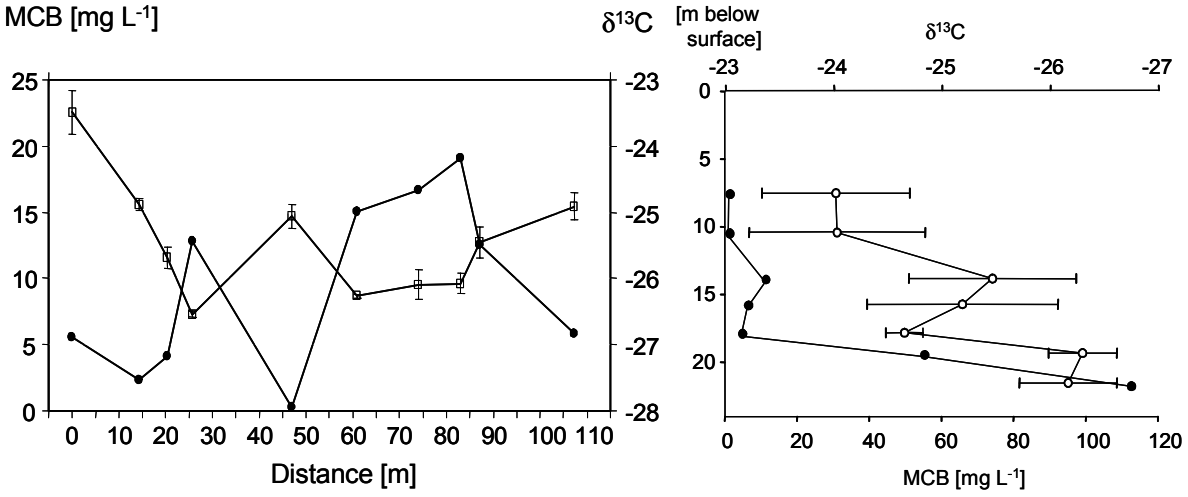


Fig. 4-8: Monochlorobenzene concentrations and isotopic signatures of the cross-section A – B, depth of 17-21 m (left) and the vertical profile in Saf 4 (right); (modified from Kaschl et al., 2005); ● = MCB concentrations; □ and ○ = $\delta^{13}C$ of MCB

Several degradation pathways are conceivable under anaerobic conditions. MCB may be degraded similarly to other aromatic compounds by processes such as nitrate-, Fe(III)-, sulfate-reduction, or fermentation coupled to methanogenesis (Chakraborty and Coates, 2005; Heider et al., 1999; Spormann and Widdel, 2000; Ulrich et al., 2005; Widdel and Rabus, 2001). In addition, microbial reductive dechlorination of MCB requiring an electron donor may also be considered. Batch experiments using several aerobic pure cultures of bacteria showed that the known aerobic pathway initiated by dioxygenases did not result in a

significant carbon isotopic fractionation (Kaschl et al., 2005). Conversely, the field isotope fractionation factor for MCB obtained in the anoxic aquifer was higher, especially if dispersion and dilution effects are accounted for (Kaschl et al., 2005). Thus, it is evident that in this aquifer a novel anaerobic pathway resulting in an isotopic fractionation which is untypical for aerobic MCB transformation must be considered to be the predominant process of the *in situ* MCB degradation. However, the present data do not allow the anaerobic pathway of MCB degradation to be identified. The transformation of MCB appears to be very slow and most of the microbial activity appears to be located at the plume fringes.

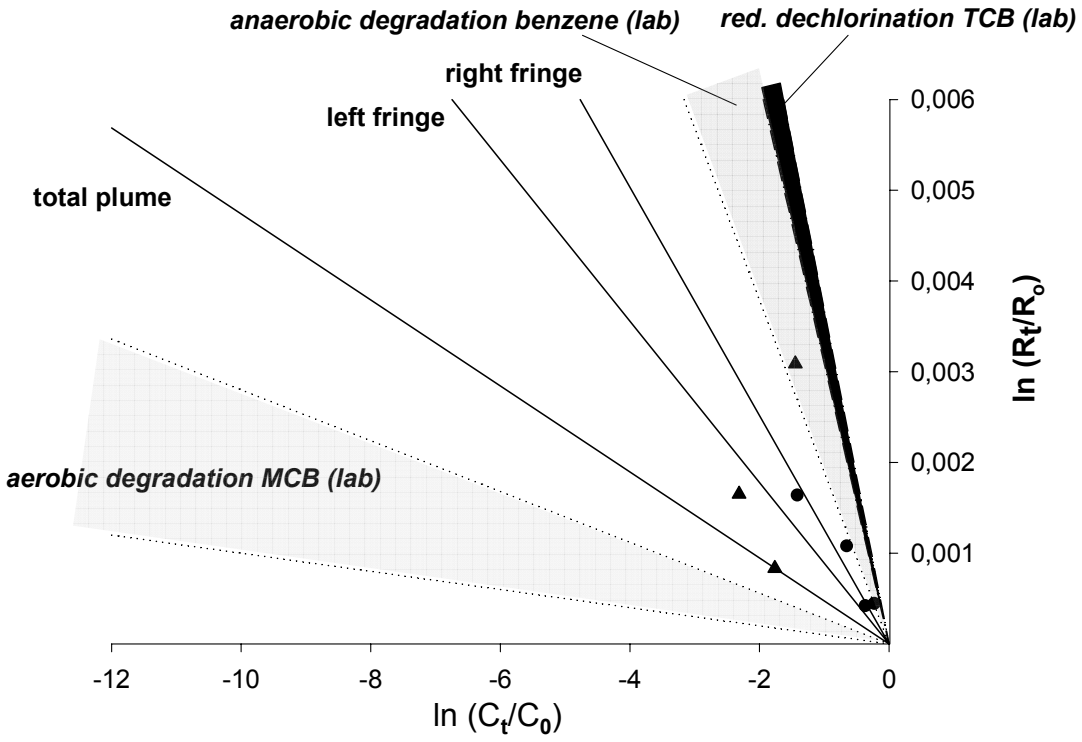


Fig. 4-9: Rayleigh plot illustrating field-derived and laboratory-derived fractionation factors: aerobic degradation of MCB in batch experiments (α=1.0001-1.00028), the field fractionation factor for the whole MCB plume (α_f=1.00043), the (▲) left (Saf11, Saf12, Saf27, Saf28) and (●) right fringe (Saf7, Saf30, Saf31, Saf23) of the plume at the cross-section A - B (α_f=1.00083 and 1.00106, respectively) and, for comparison, batch experimental data for the reductive dechlorination of TCBs (α=1.0031-1.0037 (Griebler et al., 2004a) and anaerobic degradation of benzene (α=1.0019-1.0036 (Mancini et al., 2003)).

Fig. 4-9 illustrates the various fractionation processes likely for the MCB degradation in comparison to the field data. Since these field-derived fractionation factors for MCB are significantly higher than the laboratory-derived factors for aerobic degradation, it is evident that an anaerobic pathway governs the MCB fractionation in the Bitterfeld aquifer. All known aerobic microbial cultures investigated used the ring-dioxygenase pathway to degrade MCB (Vogt et al., 2004) but the dioxygenase pathway did not lead to a significant isotope fractionation of MCB in batch experiments. This result is in accordance with studies using

toluene and naphthalene as model compounds showing that the dioxygenase reaction does not lead to significant carbon isotope fractionation (Morasch et al., 2002). Aerobic degradation of TCB by pure cultures exhibiting the dioxygenase reaction also did not show any significant isotope fractionation (Griebler et al., 2004a). However, the field fractionation factors obtained are lower compared to laboratory experiments investigating the anaerobic degradation of benzene or 1,2,3-trichlorobenzene (TCB) (Griebler et al., 2004a; Mancini et al., 2003). This may be explained by dilution, dispersion and sorption processes in the aquifer, which affect concentrations (C_t) without altering the isotopic composition (R_t). Hence, when using the Rayleigh equation (Eq. 4-2) for field data, the resulting field isotope fractionation factor is always lower than determined in culture experiments. However, in a cross section perpendicular to the contaminant plume, the dispersion and sorption processes should have affected MCB concentrations in a similar way, since the distance from the original source of the contamination is comparable. Accordingly, the fractionation factor determined at the left and right fringe was higher than that of the whole plume and was significantly higher than the low isotopic fractionation expected from aerobic transformation. The anaerobic degradation of various aromatic hydrocarbons led to a significant isotope fractionation of about α=1.0017 to 1.0036 (Mancini et al., 2003; Meckenstock et al., 1999; Morasch et al., 2001). In addition, a number of studies have demonstrated that the anaerobic reductive dehalogenation of halogenated ethenes and TCBs is always associated with higher isotope fractionation factors (Griebler et al., 2004a; Griebler et al., 2004b; Hunkeler et al., 1999a; Hunkeler et al., 2002). Analogously, it can be expected that a reductive dehalogenation of MCB should also be associated with a significant isotope fractionation. Alternatively, MCB may be degraded similarly to benzene under anaerobic conditions. Although the anaerobic degradation pathway for benzene has not yet been completely identified, the isotope fractionation of benzene yields fractionation factors of 1.0020 (methanogenic), 1.0036 (sulfate-reducing) and 1.0022-1.0024 (nitrate-reducing conditions) in enrichment cultures (Mancini et al., 2003). A degradation of MCB following the anaerobic benzene pathway should therefore also show a significant isotope fractionation.

***In situ* microcosms with stable isotope labeled substrates (BACTRAP approach)**

The *in situ* approach based upon microcosms amended with ^{13}C-labeled tracer compounds was also applied for MCB in the aquifer in Bitterfeld. The accumulation of MCB in anoxic aquifers initially led to the assumption, that anaerobic biodegradation of MCB is difficult or even impossible, because neither pure nor enrichment cultures and microcosm studies had demonstrated biodegradation of MCB under these conditions. However, the isotopic signatures of the contamination plumes clearly show biotransformation in the course of the MCB plume (Kaschl et al., 2005). BACTRAPs amended with MCB were incubated for 7 weeks in groundwater well Saf 4/97 equipped with a MLPS in order to avoid any oxygen

access changes in redox conditions. Geochemical analysis led to the conclusion that anaerobic conditions prevailed in the well where the BACTRAPs were tested.

After exposition for 49 d, more than 80% of ^{13}C-MCB had been lost from the microcosms and the extracted total lipid fatty acids were found to be significantly labeled (Fig. 4-10). Hexadecanoic acid (16:0) showed the highest incorporation of ^{13}C with δ^{13}C of 579 ‰ (Fig. 4-10). Octadecenoic acid (18:1) was also labeled significantly to 158 ‰. Octadecanoic acid (C18:0) and tetradecanoic acid (14:0) showed a lower incorporation of ^{13}C of 10 ‰ and 46 ‰, respectively. Unsaturated hexadecenoic acid (16:1) showed a strong ^{13}C signal indicating high label incorporation but the concentration was also too small for quantitative analysis. Similar to the *in situ* microcosm studies with benzene and toluene, the data provided evidence for the assumption of an unknown anaerobic MCB degradation process actually occurring in Bitterfeld. In addition, the incorporation of the label into marker molecules of the biomass also clearly indicates that this pathway supports growth and energy conservation from MCB for the respective bacteria under the given environmental conditions.

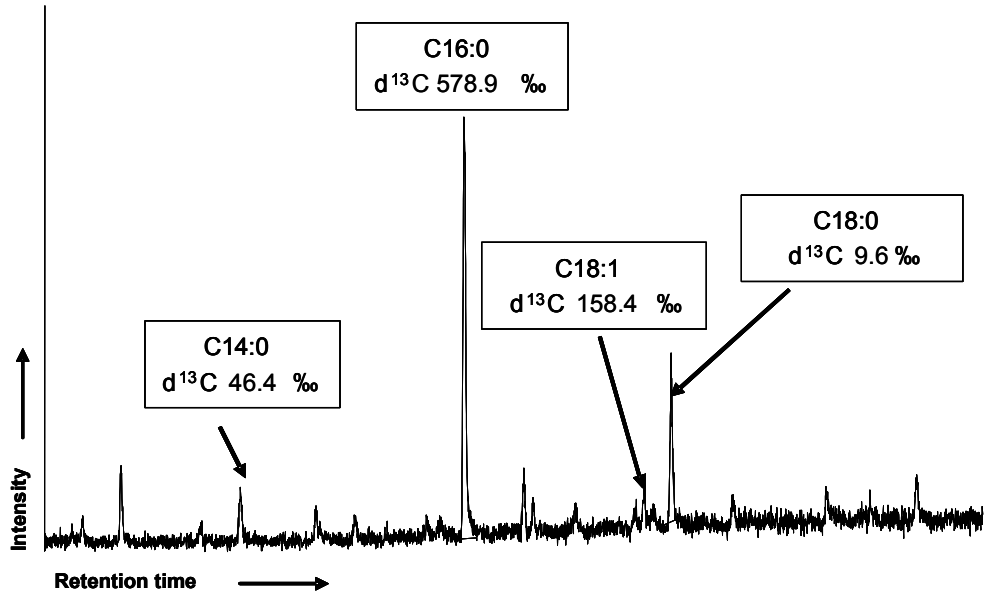


Fig. 4-10: Total ion current of the fatty acid extracts from a BACTRAP amended with [$^{13}C_6$] monochlorobenzene exposed for 49 d in an anaerobic aquifer in well Saf 4/97 at the Bitterfeld field site. Identified fatty acids and their isotope compositions are indicated in the boxes.

4.5 Concluding remarks

Both approaches presented in this overview provide promising tools for the assessment of *in situ* microbial degradation activity in contaminated aquifers. They have certain implications and the evaluation of the practical benefits and limitations is currently in progress. The isotope fractionation approach interpreting the isotope signatures of contaminants provides a technique for assessing the cumulated biodegradation in an aquifer which has been occurred

on the groundwater flow path down gradient from a source of contaminants. The SIFA approach relies on the availability of a sufficient number of monitoring wells, the availability of distinct isotope fractionation factors for the respective degradation pathways and biogeochemical conditions, and a detailed knowledge about the hydrology of the aquifer. Even if no specific isotope fractionation factor is available, a qualitative estimation of the biodegradation can be made by comparison with other isotope fractionation factors of known pathways and may provide valuable information on the processes likely to occur in the investigated aquifer. However, if multiple sources of contaminants with different initial isotopic signatures contribute to a mixed plume, this approach may be too complex for reliable quantification of *in situ* biodegradation. In addition, the applicability of the approach is limited, if processes without significant isotope fractionation govern *in situ* biodegradation as shown for aerobic toluene degradation pathways catalysed by mono- or dioxygenase enzymes reacting with the aromatic ring system (Meckenstock et al., 1999).

The *in situ* microcosm approach (BACTRAP) with isotopic labeled contaminants and analysis of the isotope composition of lipid biomarkers provides an excellent tool for assessment by proving the microbial *in situ* activity in contaminated aquifers and for monitoring natural attenuation processes. This relatively simple, low-cost approach provides information in a reasonable time frame and the results are more reliable than *ex situ* approaches. Even if the details of the metabolism of a target compound are not yet known, valuable information about the presumable pathway can be obtained by this approach. In particular, if metabolites of the parent compound can be identified on the bead material of the microcosms, a clear indication for the relevant degradation pathways can be given by this approach. Future investigations will provide the basis for a quantification of the biodegradation potential by this approach. Therefore, it is necessary to investigate the sorption behavior of the bead material in the presence and absence of bacteria. The key organisms related to contaminant degradation could not yet be identified; however, this aspect of the approach is currently under investigation. Future experiments to investigate the kinetics of the colonization as traced by quantitative PCR may elucidate the development of communities and may provide indication for organisms supported by the test substrate. When the concentration of the label in the biomass is sufficiently high, stable isotope probing (SIP) can also be employed to identify organisms which use the ^{13}C-labeled contaminants as a carbon substrate (Lueders et al., 2004; Manefield et al., 2004; Whiteley et al., 2006). This may open the “black box” of subsurface microbial ecology by linking structure and function.

4.6 Acknowledgements

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5 Sensitive detection of anaerobic monochlorobenzene degradation using stable isotope tracers

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5.1 Abstract

Microbial degradation of monochlorobenzene (MCB) under anaerobic conditions was investigated using a stable isotope tracer under *in*- and *ex situ* conditions. *In situ* microcosms were incubated directly in an anoxic aquifer and carbon derived from [$^{13}C_6$] MCB was found to be incorporated into the microbial biomass. In laboratory microcosms, amended with [$^{13}C_6$] MCB, anaerobic mineralization of MCB was indicated by the production of $^{13}CO_2$ Further, recovery of the ^{13}C-label in the fatty acids confirmed the assimilation of MCB derived carbon into microbial biomass. The described approach may be applied to various other organic groundwater contaminants of concern using carbon (^{13}C) as well as other stable isotope tracers, such as nitrogen (^{15}N), allowing direct and sensitive detection of biodegradation.

5.2 Introduction

Monochlorobenzene (MCB) is a widespread groundwater contaminant found at many former chlorine chemistry sites particularly when chlorinated pesticides have been produced [www.who.int/en/]. The Bitterfeld/Wolfen region in Germany, is a former production site for lindane (hexachlorocyclohexane) where today MCB is present as an ubiquitous contaminant throughout the entire aquifer with concentrations up to 30 mg L^{-1} (Heidrich et al., 2004a; Heidrich et al., 2004b; Popp et al., 2000). While MCB contamination may be the result of a spillage event, it can also be produced *in situ* by microbial lindane degradation or during dechlorination of higher chlorinated benzenes (Adrian et al., 2000a; Adrian et al., 2000b; Boyle et al., 1999; Fennell et al., 2004; Van Agteren et al., 1998; Van Doesburg et al., 2005). MCB is the most mobile chlorobenzene due to its relatively high solubility and lower sorption potential to the aquifer matrix and usually long plumes form indicating its high persistence in anaerobic aquifers. Drinking water limits for MCB in the USA are currently set at <0.1 mg L^{-1} [www.atsdr.cdc.gov/toxfaq], in Germany, the quality target for surface waters is set at 1µg L^{-1} conform with European Union standards [www.umweltbundesamt.de]. While aerobic degradation of MCB has been well studied (for a review see Van Agteren et al., 1998), complete anaerobic degradation and mineralization has, to our knowledge, not yet been reported. Only the complete anaerobic dehalogenation of the chlorinated benzenes to

benzene has been described but the microbiota involved in the process have not been identified (Nowak et al., 1996).

In recent years, stable isotope approaches have been developed to investigate natural attenuation of contaminants. Stable isotope fractionation analysis may be used to investigate *in situ* biodegradation of pollutants (Meckenstock et al., 2004a), additionally, stable isotopes may be applied as tracer compounds (Geyer et al., 2005; Kästner et al., 2006; Peacock et al., 2004; Stelzer et al., 2006a). Previously, evidence for the biodegradation of MCB in an anoxic aquifer in Bitterfeld was provided applying isotope fractionation techniques (Kaschl et al., 2005).

Recently, *in situ* microcosms (BACTRAP®), applying stable isotope tracers, were developed and used to investigate degradation of BTEX compounds in the field (Geyer et al., 2005; Peacock et al., 2004; Stelzer et al., 2006a). These *in situ* microcosms consist of a sorbent material serving as a surface for microbial growth and as a source for the isotopically labeled and unlabeled test substrates. During incubation of these microcosms in an aquifer for several months, microorganisms capable of degradation of the contaminant will incorporate carbon derived from the substrate into their biomass (e.g. fatty acids) and the biodegradation of the contaminant can be proven if the ^{13}C-label is recovered from the biomass. A preliminary investigation applying these *in situ* microcosms loaded with [$^{13}C_6$] MCB supported the stable isotope fractionation analysis providing evidence for the biodegradation of MCB under field conditions (Kästner et al., 2006).

In this study, stable carbon (^{13}C) tracer methods were applied to investigate the biodegradation of MCB under anoxic conditions. *In situ* microcosm systems were incubated directly in the groundwater at the field site in parallel to a laboratory set up. Production of ^{13}C-labeled degradation products and incorporation of the ^{13}C into biomass were used to demonstrate anaerobic biodegradation of MCB.

5.3 Materials and Methods

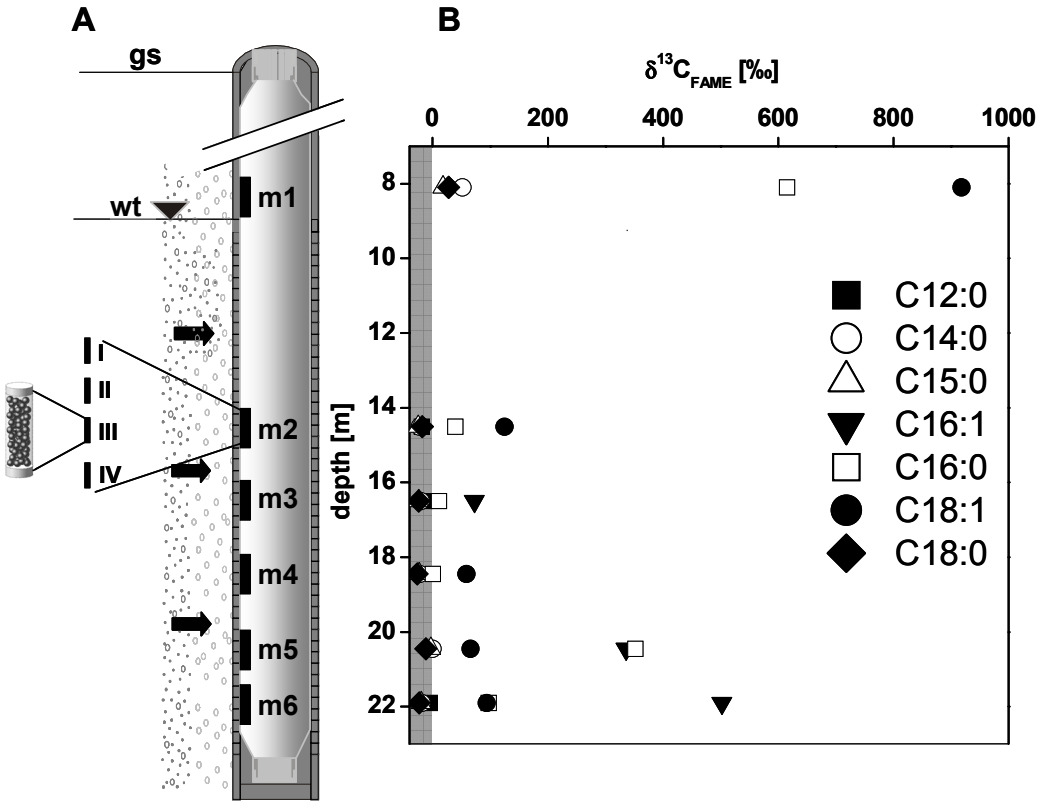
5.3.1 Chemicals

The [$^{13}C_6$] chlorobenzene was purchased from Chemotrade Leipzig (Germany) with chemical and isotopic purity of >99 %. The [$^{13}C_6$] chlorobenzene preparation further contained an impurity of 0.02% [$^{13}C_6$] benzene. All other chemicals were obtained in p.a. quality or higher.

5.3.2 Site description of the aquifer in Bitterfeld (Saxony-Anhalt, Germany)

The chlorobenzene (MCB) contaminated aquifer is located near the city of Bitterfeld, Germany. Concentrations up to 30 mg L^{-1} of MCB and lower concentrations of chlorinated aliphatic compounds (up to 5 mg L^{-1}), benzene (up to 0.77 mg L^{-1}), and dichlorobenzenes (up to 3 mg L^{-1}) were found in the Quaternary aquifer (Kaschl et al., 2005). The time of spillage is

not known but chlorobenzenes have been produced at the industrial site since the first decade of the last century. The hydrogeology and geochemistry of the aquifer system was described previously (Kaschl et al., 2005). Oxygen concentrations in the aquifer were analyzed using a flow-through chamber and an oxygen electrode (CellOx 325, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) and were generally below 0.3 mg L^{-1}, except for the highest level, close to the groundwater table where oxygen concentrations were around 1 mg L^{-1}. Using this method, oxygen concentrations below 0.5 mg L^{-1} could not be resolved accurately. Significant nitrate concentrations were only found in the upper layers of the aquifer indicating that nitrate reduction was not a relevant terminal electron acceptor (TEA) in the deeper layers of the investigated aquifer. Some iron and manganese reduction was indicated by low Fe(II) and Mn(II) concentrations. Elevated sulfide concentration associated with sulfate depletion indicated sulfate reduction and sulfate concentrations up to 1.48 g L^{-1} suggested that sulfate was available as the principle TEA in this aquifer (Kaschl et al., 2005). Elevated methane concentrations were not found in the aquifer so methanogenesis apparently was not an important process. Overall, the geochemical analysis points at prevalently anoxic conditions in the aquifer.



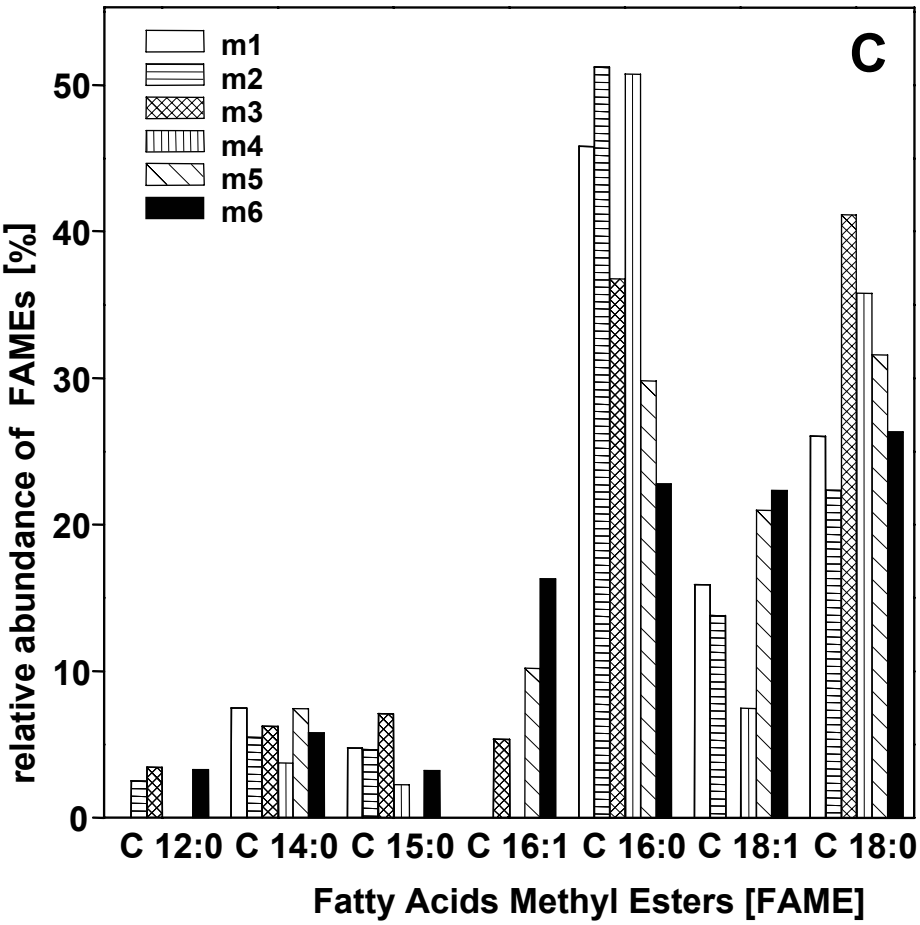


Fig. 5-1: A). Multilevel sampling system (MLPS), preventing groundwater circulation and penetration of oxygen from the surface, used for the *in situ* microcosm experiment in well SAF4. The black rectangles indicate the location of the *in situ* microcosms (m1-m6) in general between the filter screen of the well and the membrane of the MPLS, whereas the small rectangles (I-IV) show the detailed experimental set up with [^{12}C] MCB (I), Blank (II), [$^{13}C_6$] MCB (III) and Blank (IV) *in situ* microcosms as it was performed in every depth. The black arrows show the groundwater flow direction, gs: ground surface and wt: water table. B). Carbon isotope composition of fatty acid methyl esters (FAME) extracted from [$^{13}C_6$] MCB amended *in situ* microcosms incubated for 7 weeks at different depth (m1-m6) at the multilevel monitoring well SAF4 in Bitterfeld. The grey shaded area indicates the natural abundance of $\delta^{13}C_{FAME}$. C) Relative abundance of total lipid fatty acids [FAME] extracted from *in situ* microcosm loaded with $^{13}C_6$-labelled MCB incubated in different depths (m1-m6) at the monitoring well SAF4 in Bitterfeld.

5.3.3 Preparation and incubation of *in situ* microcosms

The *in situ* microcosms were prepared as described before by Stelzer et al. (2006a). Briefly, Bio-Sep® beads (University of Tulsa, Tulsa, USA) were loaded with MCB as carbon substrate for microorganisms via gas phase under reduced pressure. The *in situ* microcosms for the experiments in Bitterfeld were amended with [$^{13}C_6$] MCB or natural abundance MCB to a concentration of 76 mg g^{-}1 sorbent material. In parallel, unamended *in situ* microcosms (blank) were deployed. Material from these control experiments was used to investigate the isotope composition of fatty acids at natural abundance. The *in situ* microcosms were

incubated for 7 weeks in groundwater well SAF4 equipped with a multilevel sampling device (MLPS) to avoid any oxygen penetration and groundwater circulation between depths (Fig. 5-1; Fig. S1 Supporting Information (Anhang E)) (Schirmer et al., 1995). In the MLPS experiments the microcosms were placed in stainless steel cages as a spacer to ensure a permanent flow of ground water through the *in situ* microcosms and to avoid contamination with biological material by direct contact with the packer membrane or the filter screen of the well. The set of *in situ* microcosms used as an inoculum for the enrichment cultures were incubated directly in the groundwater in the wells without a MLPS or stainless steal cages.

5.3.4 Extraction and derivatization of fatty acids (FA)

After removal of the *in situ* microcosms from the groundwater well, the Bio-Sep® beads were extracted using a dichloromethane-methanol-water mixture as solvent modified after Blight et al. (1959). For investigation of the laboratory microcosms and enrichment cultures, 15 or 3 ml of liquid, respectively, was removed from the microcosms for extraction with the dichloromethane-methanol mixture. After phase separation the dichloromethane phase containing the fatty acids (FA) was evaporated to dryness and derivatized using a trimethylchlorosilane (TMCS) methanol mixture (1:8; v:v) as reactant for 2 h at 70 °C to obtain total lipid fatty acid methyl esters (FAME) (Thiel et al., 2001). After evaporation to complete dryness the FAME fraction was dissolved in n-hexane for subsequent analysis using a gas chromatograph coupled to a mass spectrometer (GC-MS) and a gas chromatography-combustion-isotope-ratio-monitoring-mass-spectrometer system (GC-C-IRMS) (See Supporting Information, Anhang E). FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco, Munich, Germany) and concentrations of FA were quantified relatively to the internal standard (Henicosanoic acid, C21:0). The fatty acids are designated in the form of A:B where A is the number of carbon atoms, B is the number of double bonds.

5.3.5 Laboratory microcosms

Microcosms

Groundwater was taken from 2 wells (SAF11 and SAF30) at the MCB contaminated field site in Bitterfeld. The filter screens of the wells are at 19 to 20 and 19 to 21 meters below ground, respectively. After transport to the laboratory and transfer into an anaerobic glovebox (98-96% N2/ 2-4%H2), the groundwater was amended with 1 mg L^{-1} of resazurin as a redox indicator (Tratnyek et al., 2001). The microcosms were prepared in 120 mL vials to which 100 mL of the groundwater mixture was added. The vials were closed with Teflon coated grey butyl rubber stoppers and crimped with aluminum crimps. For each well, 11 microcosms were prepared. Of each set, 2 microcosms were autoclaved for 40 minutes on three consecutive days to prepare killed controls. Then, 5 of the live microcosms and the 2 killed

microcosms were amended with natural abundance MCB and the other 4 with [$^{13}C_6$] MCB to a concentration of approx. 100 µmol L^{-1}. MCB concentrations were followed by GC-FID analysis. CO_2 concentrations were estimated by GC-C-IRMS vs. an external calibration curve. SAF11 and SAF30 contained 6.7 and 3.7 mmol L^{-1} CO_2 respectively. For details of the analysis see Supporting Information (Anhang E). Sulfide concentrations were determined according to the method of Cline (1969). Iron(II) concentrations were determined by the Department of Analytics at the UFZ-Centre for Environmental Research using the Spectroquant® Iron test (Merck, Darmstadt, Germany).

***In situ* microcosm enrichment**

In situ microcosms loaded with natural abundance MCB from a preliminary experiment incubated for approx. 8 weeks in the groundwater wells SAF11 and SAF30 were used as an inoculum for the enrichment cultures. Therefore, the *in situ* microcosms were recovered after incubation and the sorbent material was added to 27 mL anaerobic culturing tubes filled with groundwater from the monitoring wells. The tubes were closed with Teflon coated grey butyl rubber stoppers and crimped. In the laboratory, the tubes were amended as follows with anoxic sterile stock solutions to a final concentration of: 1 mg L^{-1} resazurin, 2 mg L^{-1} yeast extract, 2 mg L^{-1} vitamin B12, 1 mM Na_2S and either, for the sulfidogenic enrichments 5mM $FeSO_4$ or, for the methanogenic enrichments, 2 mM $FeCl_2$. Approx. 20 µmol MCB was added to each tube. Due to sorption of MCB to the Bio-Sep® beads, it was not possible to quantify MCB in these enrichment cultures. Qualitatively, MCB appeared to decrease over time and, apparently, microbial conversion occurred (data not shown). After incubation of approx 4 month, sub cultures were made from each *in situ* microcosm enrichment. Liquid from each microcosm was diluted (10%) into fresh basal medium according to the description by Zinder (Zinder, 1998) amended with 0.1 % $NaHCO_3$, vitamin solution, 1mM Na_2S and, as indicated 5mM $FeSO_4$ or 2 mM $FeCl_2$. Two parallels were prepared for each transfer set: one culture was amended with natural abundance MCB while the 2nd culture was amended with [$^{13}C_6$] MCB to a concentration of 500 µmol L^{-1}.

5.4 Results and Discussion

5.4.1 *In situ* microcosms

The recently developed *in situ* microcosms were directly incubated in the anoxic aquifer in Bitterfeld to investigate the MCB degradation (Geyer et al., 2005; Peacock et al., 2004; Stelzer et al., 2006a). Previous studies applying isotope fractionation techniques and a preliminary *in situ* microcosms study provided first evidence that MCB was degraded within this aquifer (Kaschl et al., 2005; Kästner et al., 2006). The *in situ* microcosms were installed inside a monitoring well in a multilevel fashion (microcosm set m1-m6; see Fig. 5-1A). The

use of a multilevel sampling device allowed depth discreet sampling and should prevent circulation of groundwater between the different levels and entry of oxygen to the microcosms during exposure (Schirmer et al., 1995). However, water may circulate between different levels through the filter material of the well screens or the surrounding sediments. After incubation in the groundwater for 7 weeks, the *in situ* microcosms were removed from the monitoring well and used for further analysis. Direct extraction of the sorbent material from the *in situ* microcosm showed that in addition to the parent [$^{13}C_6$] MCB, ^{13}C-labeled benzene was present at several of the investigated depths (m2, m3, m5, m6) with an isotope signature of $\delta^{13}C$ >1000 ‰ (Tab. 5-1).

Tab. 5-1: Carbon isotope composition of benzene and MCB extracted from blank, unloaded, and [$^{13}C_6$] MCB loaded *in situ* microcosms incubated for 7 weeks at different depths at the multilevel monitoring well SAF4 in Bitterfeld. n.a.= not analysed. The standard deviation for the analysis of the carbon stable isotope composition was <1‰ with $\delta^{13}C$<500 ‰ and between 5-10 ‰ with $\delta^{13}C$ >500 ‰.

	Blank	Blank	Labeled
Depth [m]	**$\delta^{13}C_{Benzene}$ [‰]**	**$\delta^{13}C_{MCB}$ [‰]**	**$\delta^{13}C_{benzene}$ [‰]**
m1 (8.1)	3590	190	n.a.
m2 (14.5)	1000	265	1310
m3 (16.5)	485	245	3940
m4 (18.5)	1080	50	n.a.
m5 (20.5)	700	60	8520
m6 (21.9)	560	-25.1	3780

^{13}C labeled benzene was also observed in blank, non-amended microcosms. At depth m6, ^{13}C-labelled benzene ($\delta^{13}C$ = 560 ‰) and MCB with an isotope composition typical for this aquifer section ($\delta^{13}C$ = -25 ‰) was observed in the blank, non-amended microcosms. Both compounds must have been adsorbed during incubation on the non amended microcosm from the surrounding groundwater and were present in concentrations in a similar order of magnitude, benzene concentrations were approx. one third that of MCB. (See Fig. S2, Supporting Information (Anhang E)). Although [$^{13}C_6$] benzene was already present in trace concentrations (0.02 %) in the commercial [$^{13}C_6$] MCB, it is unlikely that the labeled benzene was derived from the impurity of the chemical. Since benzene and MCB have similar physico-chemical properties, it would be not probable that a separation of both chemicals would occur upon diffusion or transport with the groundwater resulting in sorption of labeled benzene without the co-transport of labeled MCB. The results suggest that benzene is

formed upon dehalogenation of MCB in the aquifer. Additionally, at the field site, benzene could be detected in the aquifer in low concentrations (up to 20 µg L^{-1}).

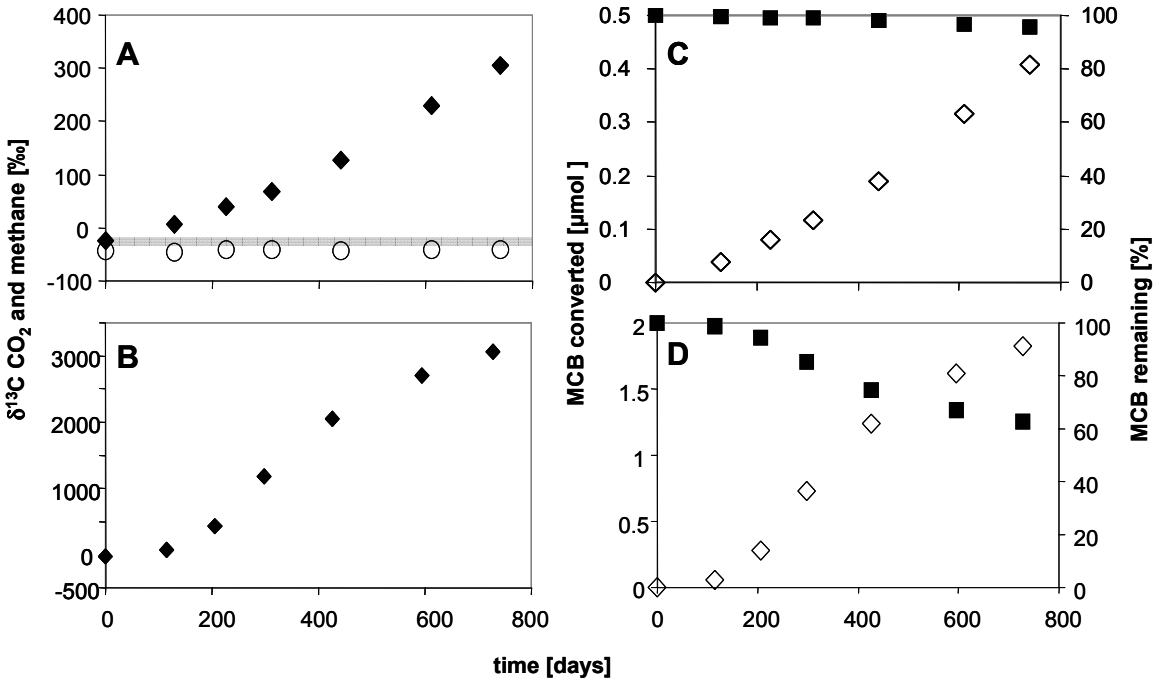


Fig. 5-2: Carbon isotope signature of CO_2 (♦) and methane (○) in A) a microcosm prepared with groundwater from SAF11 and B) the enrichment culture. Based on the CO_2 content and isotope signature, the amount of [$^{13}C_6$] MCB degraded was calculated in µmol per microcosm (◊) and % remaining of the total added [$^{13}C_6$] MCB (■) for C) the microcosm and D) the enrichment culture. The shaded area in panel A indicates the observed isotope signature of CO_2 in the control microcosms.

To investigate assimilation of MCB derived carbon into bacterial biomass, the isotope composition of fatty acids (FA) was determined. FA extracted from the *in situ* microcosm systems loaded with [$^{13}C_6$] MCB were significantly labeled providing evidence for the complete degradation of MCB via central metabolism and assimilation of MCB derived carbon for synthesis of biomass (Fig. 5-1B). The relative abundance of the various identified FA was similar comparing the microcosms incubated at the different depths m1 to m6 (Fig. 5-1B). C16:0 and C18:0 were the most abundant FA, followed by lower amounts of the mono-unsaturated C16:1 and C18:1. Only minor amounts of C12:0, C14:0 and C15:0 were detected (Fig. 5-1C). Close to the groundwater table (m1) all identified FA were labelled. Possibly, at the transition to the capillary fringe oxygen (1 mg L^{-1} at m1) is available as electron acceptor for degradation in this zone (Kaschl et al., 2005); therefore MCB may be degraded aerobically at the upper level. At deeper levels (m2-m6), conditions were strictly anaerobic and significant enrichment in ^{13}C was observed in the hexadecanoic acid (C16:0; up to $\delta^{13}C$ = 352 ‰ at depth m6); hexadecenoic acid (C16:1; up to $\delta^{13}C$ = 503 ‰ at depth m5); and the octadecenoic acid (C18:1; $\delta^{13}C$ = 94 ‰ at depth m6) (Fig. 5-1A, B). Other FA were not labelled significantly. The incorporation of ^{13}C derived from [$^{13}C_6$]-MCB into FA

provided clear evidence for *in situ* biodegradation of MCB under the environmental conditions present in the aquifer.

5.4.2 Laboratory microcosms and enrichments

Laboratory microcosms were used in parallel to investigate anaerobic MCB degradation in a closed system and to attempt cultivation of MCB degrading microorganisms. A first set of laboratory microcosms was prepared from a mixture of groundwater derived from the same MCB plume as described above. A decrease in MCB concentration was observed in several microcosms amended with electron donors (e.g. lactate, benzoate) or acceptors (sulfate), but no intermediates or degradation products were found and the activity could not be sustained (data not shown). For this reason in a second set up of microcosm systems, we supplied isotopically labeled MCB to enable a more sensitive detection of the degradation processes. Two separate sets of microcosms were prepared with groundwater from wells SAF11 and SAF30 and ^{13}C-ring labeled MCB was used as a tracer substance. No MCB decrease relative to the killed control could be observed (data not shown). The concentrations and isotope signatures of benzene, methane and CO_2 were monitored over time. In Fig. 5-2A an example is given of the observed isotope signatures for methane and CO_2 in the investigated time frame. The concentrations of CO_2 did not change significantly over the investigated period (data not shown), but the CO_2 became clearly enriched in ^{13}C (Fig. 5-2A). Over time, the isotope signature of CO_2 increased from δ= -24.4 to +305‰ in the microcosms prepared from groundwater of well SAF11 (Fig. 5-2A). The isotope signature of CO_2 in the microcosms prepared with groundwater of well SAF30 changed similarly (δ= -25.3 to +443‰). All replicates of microcosms in both sets (SAF11, SAF30) showed enrichment of the ^{13}C-label in the CO_2 fraction although the lag period for the degradation was highly variable (data not shown). After 700 days of incubation, all microcosms amended with [$^{13}C_6$] MCB had significantly produced $^{13}CO_2$. Production of $^{13}CO_2$ was not observed in any of the killed controls amended with [$^{13}C_6$] MCB. The enrichment of ^{13}C in CO_2 clearly showed the mineralization of MCB in these microcosms. Additionally, the isotope signature of methane remained stable around δ= -40 to -45‰ and δ= -45 to -50‰ for microcosms from well SAF11 and SAF30 respectively and no methane production was observed. Apparently, methanogenesis was not an important process involved in MCB degradation in our laboratory microcosms. Resazurin was added to the microcosms allowing visual monitoring of anoxic conditions during the incubation time. All microcosms remained clear throughout the incubation period indicating a reduction potential of < -0.051 mV (Tratnyek et al., 2001). At the end of the study period, soluble Fe(II) and total sulfide was measured to confirm the presence of anoxic conditions and redox buffers in the system (See Tab. S1, Supporting Information (Anhang E)). Sulfide concentrations were in the same order of magnitude,

generally a bit higher, compared to the original groundwater while soluble Fe (II) concentrations were lower confirming anoxic conditions. It should be considered that Fe(II) concentrations could have been underestimated due to precipitation with sulfide. In these microcosms, early in the incubation period (100-300 days), low levels of ^{13}C-labeled benzene could be identified by the means of retention time using headspace analysis, potentially derived from the impurity in the labeled MCB preparation. Benzene did not accumulate and disappeared over time suggesting its conversion in the microcosms.

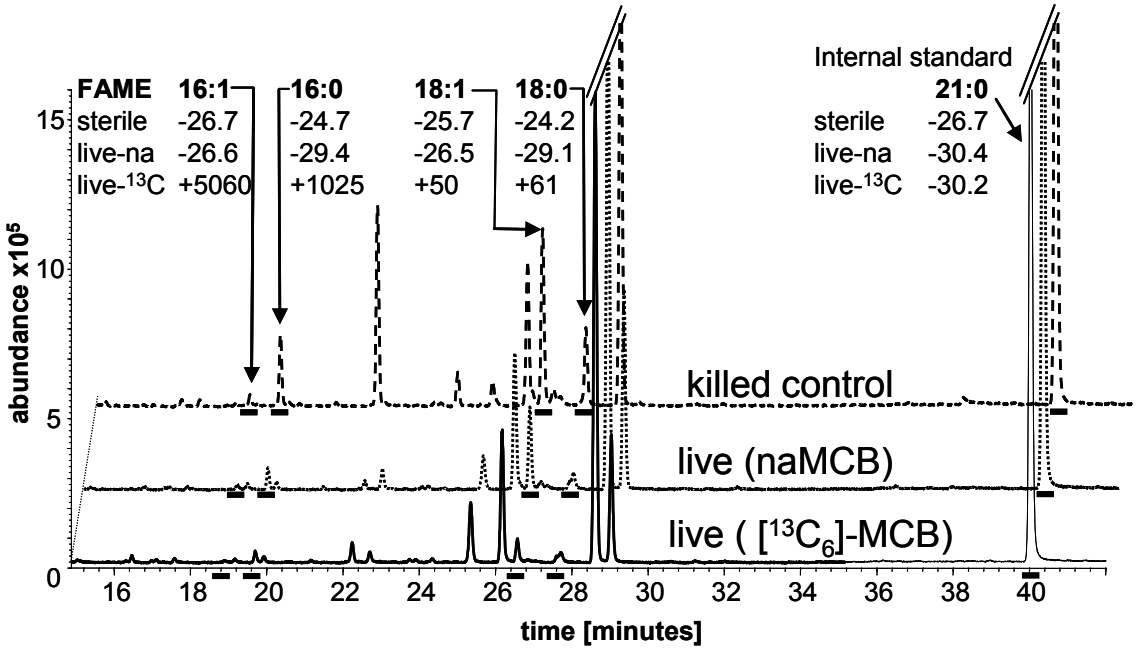


Fig. 5-3: Total ion current of fatty acid methyl esters derived from microcosms prepared with aquifer water of well SAF30. Shown are samples from a killed control, natural abundance MCB amended and [$^{13}C_6$] MCB amended microcosms. Identified peaks are labeled and underlined (_). FAME were identified via GC-MS and the isotope signature in the various samples are shown in δ‰ [PDB].

Using the concentration of CO_2 (6.7 or 3.7 mmol L^{-1} for microcosms from SAF11 or SAF30 respectively) and corresponding isotope signatures in the *ex situ* microcosms, we estimated the amount of MCB mineralized as shown in Fig. 5-2C for the microcosms from SAF11. After 700 days of incubation, 3 out of 4 microcosms from each set incubated with the labeled MCB were estimated to have converted 3 to 4% of the added MCB. Interestingly, in each set, one microcosm had converted up to 11 and 30% of MCB based on $^{13}CO_2$ evolution for microcosms derived from groundwater from well SAF30 and SAF11, respectively.

Using the analysis of the stable isotope signature of CO_2, degradation could be determined extremely sensitively. A significant enrichment in ^{13}C (e.g. δ= +50‰) would, in these microcosms from SAF11, represent an estimated degradation of about 100 nmol MCB corresponding to about 1% of the total added MCB. This order of sensitivity to measure biodegradation would be impossible to obtain using other methods except for methods using radioactive carbon tracers.

To prove incorporation of MCB-derived carbon into microbial biomass, fatty acids were extracted from the laboratory microcosms, derivatized and FA isotope signatures were analyzed. In Fig. 5-3 the total ion currents of 3 selected samples from the set SAF30 are shown. While the amount of biomass was highly similar in the different samples, [$^{13}C_6$] MCB amended microcosms showed FA which were significantly enriched in ^{13}C (16:1; 16:0; 18:0 and 18:1 with δ= +5060; +1025; +50 and +61 ‰, respectively) indicating incorporation of MCB derived carbon into biomass. Comparing the 3 samples, a shift in FA composition could be observed as octadecanoic acid (18:0) was present in the killed control microcosm but nearly absent in the live microcosms. Since the fatty acids found are common for most bacteria and have relatively low specific taxonomic value, no conclusions can be drawn about the microbial community involved in the biodegradation (Kaur et al., 2005).

According to the literature, no anaerobic MCB mineralizing microbial cultures have been described yet. In an attempt to improve the cultivability of anaerobic MCB degrading microorganisms, the sorbent from *in situ* microcosms was used as an inoculum. Therefore, after pre-enrichment of organisms during approximately 8 weeks exposure in wells SAF11 or SAF30, the *in situ* microcosms were removed and the sorbent material was used as an inoculum for enrichments. In these initial enrichments, disappearance of MCB was observed, but quantification of MCB was impossible due to the adsorption of MCB on the sorbent material resulting in only very low dissolved concentrations in the enrichment cultures. After approx. 4 months, 10% liquid transfers were prepared from these enrichments into fresh medium and [$^{13}C_6$] MCB was added as a tracer. Methane, CO_2 and benzene concentrations as well as their isotope compositions were monitored in these transfer cultures (Fig. 5-2C). The appearance of $^{13}CO_2$ was observed but, again, no labeled methane was formed. An analysis of the incorporation of carbon into biomass was also attempted. FAs were analyzed but concentrations were too low for a quantitative assessment. On the GC-C-IRMS, labeled products could be observed in the isotope trace at the same retention time as commonly found FA (C16:1; C16:0; C18:1; C18:0) implying the presence of labeled biomass (data not shown). Apparently, only small quantities of biomass were formed in our enrichment cultures which corresponded with the absence of obvious biomass formation and turbidity.

Two major hypothetical degradation pathways would come into consideration for the anaerobic biodegradation MCB: 1) initial dechlorination, followed by the degradation of benzene or 2) direct attack on the benzyl ring with the formation of a substituted chlorinated aromatic substance (e.g. chlorobenzoate, chlorophenol). The first pathway would suggest a similar pattern as observed previously by Nowak et al. (1996). Benzene could then be further degraded via benzoate or phenol as intermediates (Chakraborty and Coates, 2005; Edwards and Grbic-Galic, 1992; Lovley, 2000; Phelps et al., 2001; Ulrich et al., 2005). Alternatively, a chlorinated phenol, benzoate or toluene could be formed which then is dechlorinated via

reductive dechlorination as described for chlorinated benzoate to benzoate by e.g. *Desulfomonile tiedje* or for chlorinated phenols to phenol by *Desulfitobacterium* spp. (Löffler et al., 2003; Mohn and Tiedje, 1990; Van de Pas et al., 1999). Although the observed traces of benzene on blank *in situ* microcosms suggest degradation via dehalogenation, because of the impurity of benzene in the labeled MCB preparation the 1st pathway could not be verified in the laboratory experiments. However, the *in situ* experiments indicated that reductive dehalogenation may occur in the aquifer. Further detailed investigations of the pathway and development of a stable mixed culture and further isolation of microorganisms involved in the anaerobic MCB degradation are required.

To summarize, for the first time we could demonstrate the sensitive detection of anaerobic biodegradation of MCB, which was thus far considered persistent, *in situ* as well as in *ex situ* laboratory studies. A previous investigation supported MCB degradation in an anaerobic aquifer via stable isotope fractionation analysis but no definite proof for anaerobic degradation was presented thus far (Kaschl et al., 2005). By means of stable isotope tracers (^{13}C) we provided evidence for anaerobic MCB degradation although only very small portions of the contaminant (nmol) were mineralized. Our concept based on the use of ^{13}C labeled tracers in combination with *in situ* and laboratory microcosms was found very valuable to analyze the slow degradation of recalcitrant organic chemicals. The application of modern analytical techniques for isotope analysis enables stable isotope probing of cell components (FA), allowing the sensitive elucidation of carbon fluxes on a molecular level. Although, stable isotope probing of DNA or RNA could give valuable information about the microbial community involved in the degradation of MCB, application of these techniques in our field and laboratory systems is currently not possible yet due to the lack of biomass formed.

The described approach can be applied to detect the biodegradation and to investigate the persistence of a variety of recalcitrant environmental pollutants using ^{13}C or ^{15}N stable isotope tracers which are needed for the synthesis of cell components. Furthermore, we can use the *in situ* microcosms as pre-enrichments to improve cultivation efficiency of the microbial community involved in the degradation processes. In the future, our concept may be applied to investigate the environmental fate of recalcitrant organic chemicals providing valuable support for evaluation of chemicals for regulatory framework such as Registration, Evaluation and Authorisation of Chemicals (REACH) recently developed in Europe.

5.4.3 Acknowledgements

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Supporting Information Available

Additional information is available on the detailed methods for analysis using GC-MS and GC-C-IRMS, the analysis of the *in situ* and laboratory microcosms (Anhang E).

6 Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers

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(This chapter is submitted to Environmental Science and Technology)

6.1 Abstract

Assessment of intrinsic microbial activity under anoxic conditions at a chlorobenzene contaminated field site was addressed by hydrogeochemical analysis, stable isotope tools and multivariate statistics to elucidate the fate of chlorinated benzenes with particular emphasize on most persistent monochlorobenzene (MCB). *In situ* microcosm analysis provided evidence of microbial assimilation of MCB-derived carbon and laboratory investigations asserted mineralization of MCB at very low degradation rates. To further investigate *in situ* biodegradation of chlorinated benzenes (CBs), compound-specific stable isotope analysis (CSIA) was applied. During sequential degradation of CBs the isotope signature of a particular chlorobenzene species might be affected simultaneously by depletion and enrichment of ^{13}C, complicating the application of the Rayleigh approach for quantification of *in situ* degradation. Therefore, an isotope balance concept was applied. The enrichment of the cumulative isotope composition of all CBs indicated *in situ* biodegradation. Additionally, the linkage between geochemistry, contaminant concentration and the observed degradation activity was investigated by principal component analysis (PCA) underlining hydrogeochemical heterogeneity at the study site. The application of an integrative approach relying on multiple lines of evidence to document that capability of the intrinsic microbial community to anaerobically degrade MCB and DCB was found useful to characterize the natural attenuation potential of the site.

6.2 Synopsis

Multiple lines of evidence demonstrated *in situ* degradation of chlorinated benzenes in an anoxic aquifer.

6.3 Introduction

Chlorinated aromatic compounds are worldwide intensively used to synthesize pesticides and other chemicals leading to ubiquitous distribution in the environment (Field and Sierra-Alvarez, 2008). Chlorinated benzenes (CBs) can also be formed during anaerobic microbial transformation of hexachlorocyclohexane, of which the γ-isomer (Lindane) is worldwide used as

pesticide (Phillips et al., 2005; Van Agteren et al., 1998). Due to their toxicity, persistence and accumulation in the food chain CBs are of great environmental concern.

CBs may be subjected to both aerobic and anaerobic microbial degradation (Adrian and Gorisch, 2002; Field and Sierra-Alvarez, 2008; Van Agteren et al., 1998). With increasing chlorination CBs may undergo reductive dehalogenation (RDH) either as cometabolic reaction or energy yielding halorespiration (Adrian and Gorisch, 2002; Van Agteren et al., 1998). The occurrence of RDH under methanogenic and sulfate reducing conditions has been observed in a variety of anaerobic mixed cultures (Adrian and Gorisch, 2002; Van Agteren et al., 1998), but only one bacterial strain (*Dehalococcoides* strain CBDB1) capable to couple energy conservation with RDH of CBs (≥ 3 chlorine substituents) has been isolated so far (Adrian et al., 2000b; Jayachandran et al., 2003). RDH generally results in the transformation of higher chlorinated CBs but may lead to an accumulation of lower chlorinated CBs such as monochlorobenzene (MCB) under strongly reducing conditions. Preliminary indications for RDH of MCB have been presented (Nowak et al., 1996). Kaschl et al. suggested that anaerobic MCB degradation may lead to significant stable carbon isotope fractionation and applied compound-specific stable isotope analysis (CSIA) to further indicate *in situ* biodegradation of MCB in an anoxic aquifer (Kaschl et al., 2005). Recently, complete mineralization of MCB has been proven using stable isotope tracer techniques which enabled very sensitive detection of low rate degradation processes (Nijenhuis et al., 2007).

CSIA has been established for monitoring the biodegradation of pollutants at contaminated field sites (Meckenstock et al., 2004a). This method was successfully applied for BTEX (Fischer et al., 2007; Griebler et al., 2004b; Mancini et al., 2002; Vieth et al., 2005), fuel oxygenates (Kuder et al., 2005; Zwank et al., 2005) and chlorinated ethenes (Hunkeler et al., 1999a; Imfeld et al., 2008; Nijenhuis et al., 2005; Sherwood Lollar and Slater, 2001) but, to our knowledge, beside the work of Kaschl et al. (2005) no further field studies exist for CBs. Moreover, most studies were conducted to characterize degradation of single contaminants instead of looking at complex scenarios involving sequential degradation mechanisms, multiple contaminants and/or various degradation pathways.

Microbial degradation of organic contaminants is associated with isotope fractionation, leading to an enrichment of heavier isotopes in the residual non degraded fraction. The magnitude of isotope fractionation mainly depends on the reaction mechanism. As shown for RDH of chlorinated ethenes (Cichocka et al., 2008; Nijenhuis et al., 2005) and trichlorobenzenes (TCB) (Griebler et al., 2004a), degradation of CBs should also be associated with significant isotope fractionation, if the reduction of a Cl-C-bond is involved. Similar to anaerobic BTEX degradation, the anaerobic oxidation of the benzene ring for which cleavage of a C-H-bond is expected should lead to isotope fractionation (Fischer et al., 2008; Mancini et al., 2003). However, if the fate of a contaminant is simultaneously controlled by its production (associated

with depletion in ^{13}C) and further degradation (leading to enrichment in ^{13}C), as expected from sequential RDH, the isotope enrichment might be masked due to opposite isotope fractionation limiting the use of the Rayleigh approach for contaminants (Hunkeler et al., 1999a; Nijenhuis et al., 2005).

At the study site, release of CBs resulted in a severe contamination of the groundwater. It is hypothesized that RDH and/or anaerobic oxidation govern the removal of CBs under anoxic conditions in the aquifer. Since active remediation technologies were recognized as technically and economically not feasible (Thullner and Schäfer, 1999), this study aimed at evaluating the efficacy of natural attenuation (NA) as remedial option.

For this purpose, in accordance with guidelines provided by the US-EPA (1999), we developed an integrative approach including (i) assessment and monitoring of CB degradation and plume stability by means of CSIA and tracer studies under field and laboratory conditions as well as (ii) characterization of hydrogeochemical conditions governing *in situ* biodegradation applying multivariate statistics. Special attention was put in interpreting MCB degradation since its accumulation seems to be the limiting factor to apply NA as remediation strategy. To overcome complications associated with the Rayleigh concept for interpretation of the isotope data, an isotope balance was calculated to assess biodegradation of CBs.

6.4 Material and Methods

6.4.1 Study Site and sampling

The field site is located at a former chemical plant where the production of mostly pesticides caused an intensive release of chlorinated organic contaminants to the subsurface. The direct source zone characterized by high loads of contaminants and dense nonaqueous phase liquid (DNAPL) was completely encapsulated and hydraulically disconnected from the surrounding natural groundwater system to prevent further release of contaminants. The initial contaminant pattern dominated by higher chlorinated benzenes is preserved inside the containment whereas a plume mainly consisting of MCB and DCB with total concentrations up 2840 µg L^{-1} has developed. A detailed site description is given in supporting information (SI) (section 1.1, Anhang F).

Groundwater samples for hydrogeochemical and isotope analyses were taken in 2005 and 2006 from 22 monitoring wells (Fig. 6-1). The sampling, extraction procedure and analytical methods are described in SI (Anhang F). A table summarizing the geochemical data collected during sampling in 2005 is given in SI (Tab. S2, Anhang F).

6.4.2 Chemicals

The [$^{13}C_6$] MCB was purchased from Chemotrade Leipzig (Germany) with chemical and isotopic purity of > 99%. All other chemicals used were obtained in p.a. quality or higher.

6.4.3 Field study with *in situ* microcosms

In February 2005 a field experiment was performed using an *in situ* microcosm test system (BACTRAP) as described elsewhere (Geyer et al., 2005; Kästner et al., 2006; Nijenhuis et al., 2007; Peacock et al., 2004; Stelzer et al., 2006a). Briefly, Bio-Sep® beads were loaded with [$^{13}C_6$] MCB to a concentration of about 100 mg g^{-1} beads. The *in situ* microcosms were deployed in 6 different wells at the low (H, L), medium (D, E) and high contaminated (A, B) area of the plume in the lower strictly anoxic part of the aquifer (Fig. 6-1 and Fig. S1, Anhang F). After 72 days *in situ* microcosms were removed from the aquifer and the carbon isotope signature of total lipid fatty acids (TFLA) was analyzed. Detailed information about the extraction procedure and analytical methods can be found in SI (section 1.4, Anhang F).

6.4.4 Laboratory study with enrichment cultures

Material of *in situ* microcosms amended with natural abundance MCB as described above were used as inoculum for the laboratory enrichment cultures. From each selected well (A, B, D, E, H, L) four enrichment cultures were prepared in 38 ml vials. Each vial finally contained approx. 27 ml of groundwater and 4-6 Bio-Sep® beads. MCB was added to the vials: always two cultures were amended with 1 µl natural abundant and another two with [$^{13}C_6$] MCB. Cultures were incubated stationary at 20°C in the dark and were sampled at regular time intervals to determine the carbon isotope signature of mineralization products (CO_2, CH_4). A more detailed description of the methods is given in SI (section 1.5, Anhang F).

6.4.5 Determination of $\delta^{13}C$ values for the total chlorinated benzenes

The isotope signatures of individual CBs ($\delta^{13}C_i$) were analyzed by GC-C-IRMS and are given in δ-notation (per mill) (for details see SI, section 1.6, Anhang F).

The isotope signature (per mill) of the total CBs ($\delta^{13}C_{CB}$) was calculated by multiplying the molar concentration of each compound (C_i) with its respective carbon isotope signature ($\delta^{13}C_i$), adding all contributions and dividing by the total molar concentration of all chlorinated benzenes (C_{CB}) (Eq. 6-1).

$$\delta^{13}C_{CB}[‰] = \frac{\sum (C_i * \delta^{13}C_i)}{C_{CB}}$$

Equation (6-1)

In this paper we refer to the term isotope balance to clearly distinguish between the calculated isotope signature of the total CBs ($\delta^{13}C_{CB}$, isotope balance) and the directly measured isotope signatures ($\delta^{13}C_i$) of the single species of CBs, such as MCB and DCB isomers.

The uncertainty associated with the isotope balance was calculated based on the error propagation using the standard deviation of each chlorobenzene species (Eq. 6-2).

Equation (6-2)
$$\Delta_{tot}\delta_{stdev} = \frac{\sqrt{\sum \left(C_i * \Delta\delta^{13}C_i\right)^2}}{C_{CB}}$$

For all samples the standard deviation for the values of the isotope balance was ≤ 0.4 ‰.

6.4.6 Principal Component Analysis

Principal component analysis (PCA) was used to condense the hydrogeochemical and isotope data into a reduced number or orthogonal linear combinations and to gain insight into the relationship between variables. The numerical data matrices were converted and the correlation analyses carried out using the program R (R: Copyright 2005, The R Foundation for Statistical Computing Version 2.1.1). PCA was performed on the correlation matrix applying the logarithmic transformation to meet normality requirements. Two data sets were subjected to PCA: (i) the hydrogeochemistry data (Tab. S2, Anhang F), isotope composition of MCB and of total CBs corresponding to the year 2005 (Tab. S4, Anhang F), as well as (ii) concentrations of MCB and DCB isomers, isotope composition of MCB and the total CBs corresponding to years 2005 (Tab. S4, Anhang F) and 2006.

6.5 Results and Discussion

6.5.1 *In situ* microcosms

The patterns of TFLA extracted from *in situ* microcosms are provided for one representative well located at the low (H), medium (E) and high contaminated (A) area of the plume (Fig. S1, Anhang F). All samples showed significant amounts of saturated hexadecanoic (C16:0) and octadecanoic (C18:0) acids as well as the unsaturated hexadecenoic (C16:1) and octadecenoic (C18:1) acids. Further, an octadienoic acid (C18:2) was identified in all samples. Comparison of the three samples revealed only minor differences in the fatty acid (FA) compositions. While sample H was dominated by C18:0, the most abundant FA in the other two samples was a C18:1 isomer. In sample A, octadecanoic acid was one order of magnitude less abundant compared to E and H (Fig. S1, Anhang F). TLFA profiles showed low taxonomic value similar to previous findings (Geyer et al., 2005; Stelzer et al., 2006a). The total concentrations of TLFA varied between 4000-12000 pmol per microcosm (data not shown) suggesting a significant microbial colonization in the bead material similar to previously published results (Geyer et al., 2005).

PLFA may offer a sensitive measure to characterize viable bacterial community structures whereas TLFA fraction can additional comprise lipids of dead biomass (Green and Scow, 2000; Kaur et al., 2005; Lu et al., 2007). Basically, the microbial community trapped on *in situ* microcosms may consist of both viable and non-living organisms as well as degraders and non-

degraders. Therefore extraction of TLFA was favoured over PLFA to investigate more sensitively the incorporation of ^{13}C into total biomass as an indicator *for in situ* activity of the bacterial community.

Compared to unlabeled controls ($\delta^{13}C_{FAME}$ = -30 ± 5 ‰, data not shown), the TLFA methyl ester fraction of labeled samples showed a clear enrichment of ^{13}C up to 4500 ‰ in individual fatty acids (Tab. 6-1). Fatty acids with 16 carbons generally represented the highest enrichment in ^{13}C and unsaturated FA were higher labeled than the saturated ones as similarly observed by Nijenhuis et al. (2007). FA with odd chain length (C17) showed the highest $\delta^{13}C$ value of 4500 ‰ which corresponded to 6 atom % incorporation, but was only found in sample A in very low quantity. For some FA (C18:2, C18:1) no label was found (-31 to -23 ‰) indicating part of the microbial community were not involved in MCB degradation.

However, the transformation of the labeled carbon from the [$^{13}C_6$] MCB into bacterial fatty acids provided evidence of microbial degradation of MCB under ambient aquifer conditions although an enrichment of ≤ 6 atom percent indicated that microorganisms colonizing the *in situ* microcosms mainly used other carbon sources than the labeled MCB. In general, the labeling was comparable in all three samples but slightly higher at A suggesting that the presence of a more MCB adapted microbial community correlated with high MCB concentrations.

Tab. 6-1: Carbon isotope signature of TLFA methyl esters $\delta^{13}C_{FAME}$ [‰] extracted from *in situ* microcosms amended with [$^{13}C_6$] MCB and incubated in the contaminated aquifer at the study site.

Well [C_{MCB} in µg L^{-1}]	A [1400]	E [430]	H [120]
C16:1	2600	2000	2000
C16:1	nd	nd	2900
C16:0	1600	70	10
iC17:0	4500	nd	nd
C18:2	-28	-31	-31
C18:1	-28	-31	-31
C18:1	360	43	1
C18:0	50	-25	-31
C21:0	-23	-26	-30

nd - not detectable

6.5.2 Laboratory enrichment cultures

To prove the potential for mineralization of MCB by the microflora colonizing the *in situ* microcosms, cultivation-dependent methods were used in the laboratory. Surfaces are very

often required for successful cultivation and pre-incubation in the field may improve the later cultivation in the lab (Herrmann et al., 2008). Therefore, material of *in situ* microcosms, which were directly incubated at the field site, served as pre enriched-inoculum. Application of ^{13}C-labeled substrates in enrichment cultures is one of the few approaches suitable to sensitively detect complete mineralization of a single contaminant in a complex mixture determining the evolution of labeled CO_2 (Morasch et al., 2007). Addition of [$^{13}C_6$] MCB allowed exclusive detection of anaerobic MCB degradation, a supposedly slow process (Nijenhuis et al., 2007).

After 197 days highest enrichment of ^{13}C in CO_2 was observed for the samples from well L with values of 629 ± 85 ‰ and lowest for wells H and D with values of 91 ± 59 ‰ and 130 ± 40 respectively. Analyses of enrichment cultures from A, B and E showed comparable results with $\delta^{13}C_{CO2}$ of 250 ± 91 ‰, 222 ± 19 ‰ and 296 ± 122 ‰, respectively (Tab. S3, Anhang F). Labeled methane could not be detected indicating that MCB degradation was not coupled to methanogenesis. All unlabeled controls showed no ^{13}C enrichment in CO_2 during the course of the experiment.

The extent of MCB mineralization was calculated according to the method of Morasch et al. (2007) based on the ^{13}C-CO_2 production during the course of the experiment in respect to the total CO_2 initially analyzed from the groundwater of the respective wells (Tab. S3, Anhang F). Mineralization rates varied between 0.1 to 1.1 nmol per day indicating very slow MCB mineralization underlining the difficulties associated with cultivation of such organisms. The results are in good agreement with data obtained from Nijenhuis et al. (2007) for a similar experiment at another field site. An electron balance to link MCB degradation to iron or sulfate reduction was not feasible due to too high background concentrations of potential electron donors (Tab. S2, Anhang F). In all samples MCB mineralization was further maintained over time up to 1000 days (data not shown). However, the underlying pathway could not be elucidated.

In summary, both the *in situ* microcosm and laboratory investigations confirmed anoxic degradation of MCB, providing proof of assimilation of MCB-derived carbon and mineralization of MCB. *In situ* microcosms provide a very promising tool to directly test *in situ* biodegradation of recalcitrant contaminants within reasonable time and this technique opens prospects for detailed analysis of the microbial key-players in future.

6.5.3 Isotope balance computation

CSIA of MCB and DCB was performed to gain information on *in situ* biodegradation within the plume and to investigate the relevance of degradation processes at the field site. Presence of these contaminants in the plume is presumably related to RDH of higher chlorinated benzenes and HCH which were initially spilled and which are still present inside the containment (SI, sections 1.1, 2.4; Anhang F).

The isotope signatures of MCB and DCB dissolved in the groundwater inside the containment were in the range of -27.7 to -29.7 ‰ and -24.0 to -27.5 ‰, respectively. In the plume DCBs showed a significant enrichment of ^{13}C compared to the containment and the isotope signatures were in the range of -25.5 to -15.6 ‰, -28.1 to -22.3 ‰ and -28.1 to -22.0 ‰ for 1,2-; 1,3- and 1,4-DCB, respectively (Tab. S4, Fig. S5; Anhang F). Assuming that due to their relatively low concentrations, RDH of TCB is not a relevant process within the plume and other sources for DCB production are lacking (SI, section 2.4; Anhang F), this high variability is supposedly related to microbial DCB degradation although no systematic correlation between concentrations and isotope signatures was observed. With increasing distance from the containment an enrichment of ^{13}C in the residual DCB fraction up to 4-5 ‰ compared to the source area was observed. Nevertheless, the highest isotopic shift (10 ‰ for 1,2-DCB and 4 ‰ for 1,3-DCB) was recorded in the area defined as contaminant source of the plume close to wells A, B, D suggesting that this part of the aquifer is probably favorable for RDH of DCB possibly associated with an accumulation of MCB. Further, our data supported the hypothesis of a preferential degradation of 1,2- over 1,3- and 1,4-DCB presumably due to higher yields in Gibbs free energy (Dolfing and Harrison, 1993) since lowest concentrations were determined for 1,2-DCB accompanied by the highest enrichment in ^{13}C and accordingly 1,4-DCB showed highest concentration and lowest isotope fractionation.

Under anoxic conditions RDH is the known degradation pathway for DCB leading to the formation of MCB, which consequently should be depleted in ^{13}C. Indeed in most of the wells the isotope signature of MCB was isotopically lighter compared to DCB. Theoretically, also a direct mineralization of DCB to CO_2 is feasible, but so far this has only been described for aerobic biodegradation (Van Agteren et al., 1998). The high abundance of MCB in the plume further indicated that apparently RDH of DCB and probably TCB led to the formation of MCB. Since no significant amounts of higher chlorinated benzenes were detectable in the plume, we suppose that the isotope signatures of DCB were only affected by its degradation.

The isotope signatures of MCB in the plume ranged between -29.6 ‰ and -25.3 ‰. Highest enrichments were found at the N and SE fringe of the plume (Fig. S5; Anhang F), where MCB concentrations are rather low. The ^{13}C-enrichment in combination with low MCB concentrations indicated MCB biodegradation in this aquifer zone. In the central and western part, presumably representing the former centerline of the plume, $\delta^{13}C$ values of MCB were more depleted indicating microbial production of MCB (Tab. S4, Fig. S5; Anhang F).

As previously suggested, two main mechanisms for MCB degradation should be considered: (i) RDH of MCB to benzene and its subsequent degradation or (ii) anaerobic oxidation of MCB which in this case would serve as electron donor (Braeckevelt et al., 2007a; Nijenhuis et al., 2007; Nowak et al., 1996). Similar to RDH of TCB or anaerobic degradation of benzene, it is expected that microbial degradation of MCB under anoxic conditions would also lead to

significant isotope fractionation (Fischer et al., 2008; Griebler et al., 2004a; Mancini et al., 2003). At the anoxic aquifer of the study site the situation is more complex: MCB could be the product of DCB dehalogenation and simultaneously be subjected to microbial degradation. Both processes are associated with an opposite isotope fractionation. The RDH of DCB may result in isotopically lighter MCB and the MCB degradation would lead to an enrichment of ^{13}C in the residual MCB fraction. For this reason the isotope signature of MCB may reflect both processes, production of MCB from DCB as well as MCB biodegradation and the Rayleigh concept is not applicable straight forward to quantify biodegradation of MCB.

In order to investigate whether or not degradation of MCB occurred, an isotope mass balance including all CBs detected was performed based on two main assumptions: transformation of DCB to MCB via RDH which is most probably associated with isotope fractionation would not influence the cumulative isotope composition of all CB ($\delta^{13}C_{CB}$) and consequently only further MCB degradation would affect the isotope balance. Therefore significant enrichment of $\delta^{13}C_{CB}$ can be considered as indicator for MCB biodegradation. Regardless of the extent of isotope fractionation of individual contaminants upon formation and degradation, the cumulative isotope composition becomes enriched upon the degradation process. This assumption is valid even if a particular degradation reaction in the reductive sequence is not associated with significant isotope fractionation. However, at least one reaction step of the reduction sequence should be linked to isotope fractionation to document biodegradation. The isotope mass balance approach is corrupted, if alternative degradation pathways, for example mineralization of a DCB isomer without formation of MBC, occur in parallel and affect concentration or isotope signature of one member of the hypothetical sequence. In this case DCBs and MCB will not form a complete reaction sequence and the enrichment of individual species reflects degradation. However, isotope enrichment of DCBs will suggest *in situ* biodegradation in any case. In addition, the isotope enrichment of MCB at the plume fringes compared to the source areas will clearly indicate *in situ* degradation. Both, the mass balance approach as well as the individual evaluation of MCB may provide evidence that NA processes governing MCB concentration. In addition, different contaminants may vary significantly concerning their physico-chemical properties which could cause a preferential transport of contaminant species between source and down gradient the plume. For DCB and MCB no considerable differences concerning their retardation are expected, since adsorption coefficients for both are almost identical (Thullner and Schäfer, 1999).

The spatial distribution of concentration and respective carbon isotope signatures for the total CBs ($\delta^{13}C_{CB}$) is presented in Fig. 6-1. The $\delta^{13}C_{CB}$ values ranged between -27.2 ‰ up to -24.8 ‰. The lowest $\delta^{13}C_{CB}$ value was found for well A, located nearby the containment and defined as the centre of the plume due to highest contaminant concentrations. The decrease in total CB concentration was associated with significant enrichment of ^{13}C by ≥ 1 ‰ (Fig. 6-2)

indicating *in situ* biodegradation. Highest ^{13}C-enrichment of total CBs was mainly observed in the west (well O and N) and north (well J) of the plume suggesting removal of CBs from the system due to biodegradation (Fig. 6-1, Fig. 6-2). Overall, with the exception of well B and I, the data obtained from the isotope balance computation indicated a destructive removal of total CBs from the aquifer and demonstrated that chlorobenzene degradation is a relevant process in most parts of the plume. Abiotic processes such as dilution were generally not expected to cause considerable isotope effects, but they can influence the contaminant concentration (Fischer et al., 2006; Kopinke et al., 2005) (Fig. 6-2).

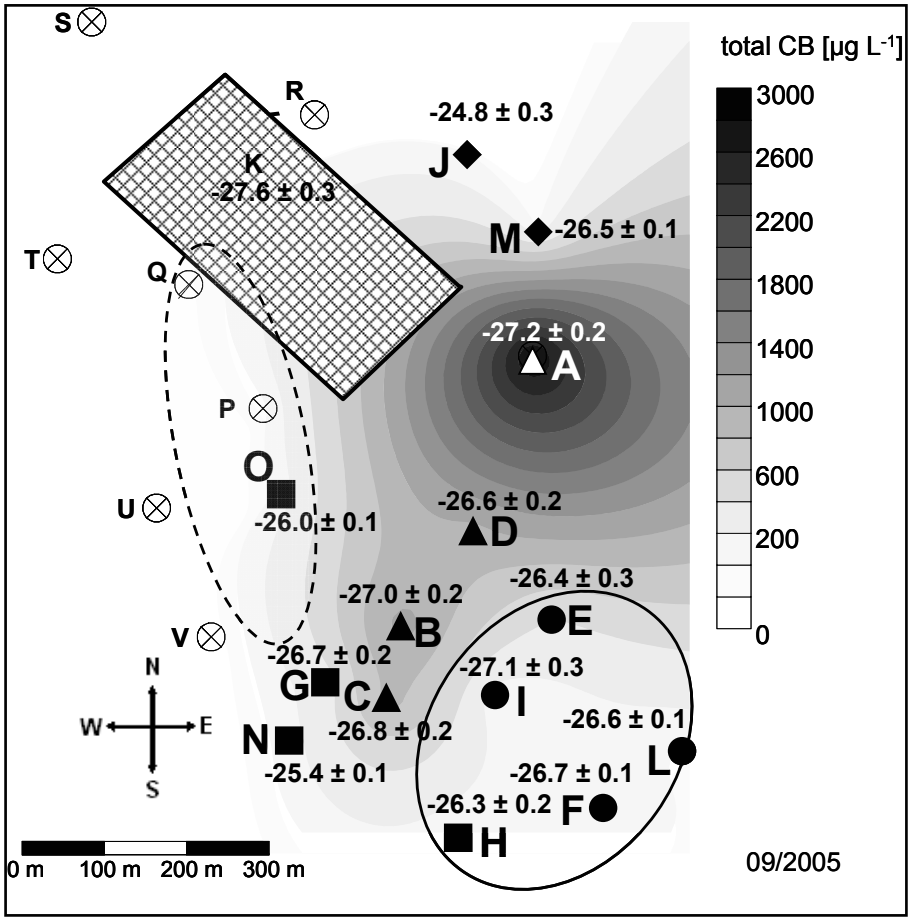

Fig. 6-1: Concentrations [µg L^{-1}] and carbon isotope signatures [‰] of total chlorinated benzenes (numbers) at the study site for sampling in 2005. The letters indicate the name of the wells and the rectangle in diagonal crosses surrounds the area of the containment, which is hydraulically disconnected from the plume. Additionally the dashed (- - -) black line shows the area were the ratio between MCB and DCB is < 0.3 and the solid (——) line indicates where the ratio between MCB and DCB is > 0.9. The symbols indicate the samples which belong to a common cluster as obtained by PCA: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M) (see Fig. 6-3). Wells which were not considered for PCA are indicated by ⊗.

The source zone (A, D, B) and the western fringe of the plume, where DCB is still present in higher amounts, were characterized by more enriched $\delta^{13}C_{DCB}$ and $\delta^{13}C_{CB}$ values suggesting microbial degradation of DCB leading to the formation of MCB more depleted in ^{13}C. MCB isotope signatures did not reflect MCB degradation in the western part of the plume. Nevertheless, the isotope balance of total CBs showed ^{13}C enrichment suggesting that

chlorobenzenes are destructively removed from the aquifer. At the SE fringe, higher $\delta^{13}C_{MCB}$ values directly indicated MCB degradation. DCB is not very abundant at this part of the aquifer and consequently significant formation of MCB due to DCB degradation is unlikely.

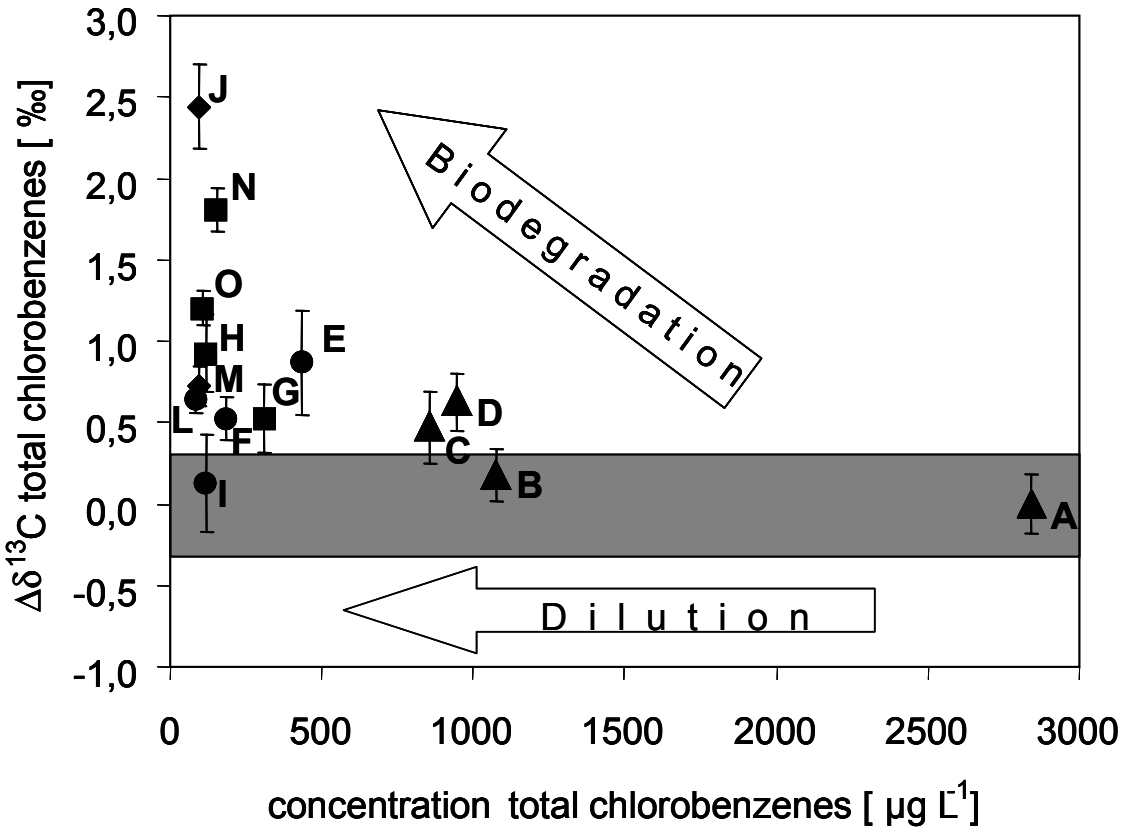

Fig. 6-2: Correlation between concentration [µg L^{-1}] and isotopic enrichment [‰] of total chlorinated benzenes. The symbols indicate the sampling locations which belong to a common cluster as obtained by PCA: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M) (see Fig. 6-3).

6.5.4 Statistical analysis of the field data

Principal Component Analysis (PCA) was used to highlight relations between hydrogeochemical patterns of various sampling locations and to support delineation of prevailing biogeochemical processes at the contaminated groundwater system (Andrade et al., 2008; Lee et al., 2001).

PCA permitted to identify factors driving the separation between clusters and consequently highlighting geochemical variables associated with changes in the isotopic signatures. In the hydrogeochemical analysis of year 2005, the percent of variance within the data accounted for by the first (PC1) and second principal component (PC2) was high (55 %; Fig. 6-3). Scores of PC1 correlated positively to $\delta^{13}C_{MCB}$, total organic carbon (TOC), electric conductivity, ferrous iron, methane and $\delta^{13}C_{CB}$ (loadings > 0.3) and negatively to total CB and MCB concentrations. This emphasizes the significance of these variables in distinguishing clusters of sampling locations. Moreover, isotopic composition values of both MCB and CBs were negatively

correlated with their concentration values (Pearson's product-moment correlation, $P<0.05$) which supported the results of the isotope balance illustrating stable isotope fractionation as a function of microbial degradation. Sampling locations experiencing degradation activity (cluster II and IV) could be distinguished from apparently less active locations (cluster III) or locations where the activity could hardly be detected (cluster I). Overall, higher PC1 and PC2 scores indicated higher biodegradation activity in a well (Fig. 6-3).

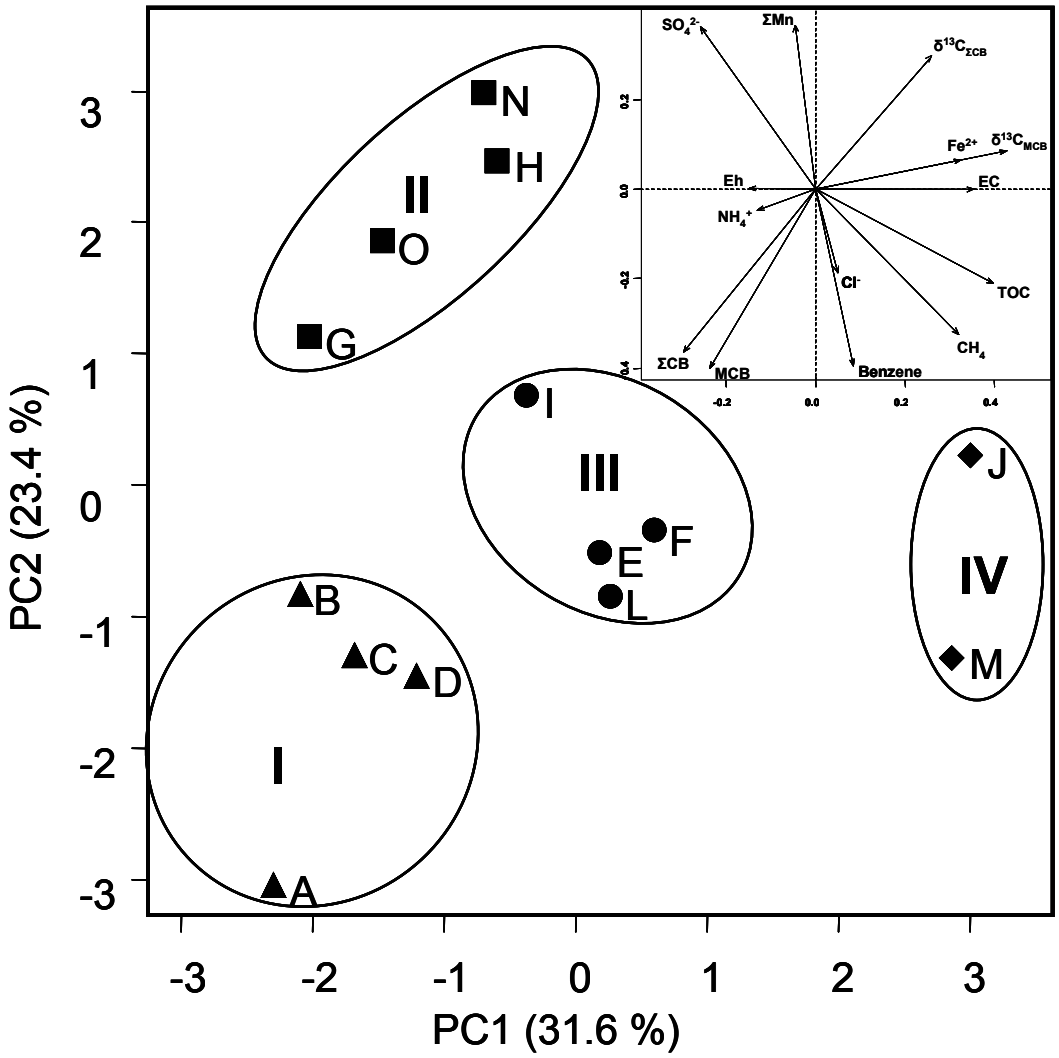

Fig. 6-3: PCA ordination plot of geochemistry, contaminant concentrations, carbon isotope composition of MCB and total CBs in groundwater samples obtained during sampling in 2005. Values on the axes indicate % of total variation explanation by the corresponding axis (PC1, principal component axis 1; PC 2, principal component axis 2). The symbols indicate the samples which belong to a common cluster: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M). The figure in the upper right corner of the plot depicts the relative contribution of the descriptors in the reduced space. Description vectors correspond to: $\delta^{13}C_{\Sigma CB}$, isotope balance of total CBs; $\delta^{13}C_{MCB}$, carbon isotope signature of MCB; Fe^{2+}, ferrous iron; EC, electric conductivity; TOC, total organic carbon; CH_4, methane; Cl^-, chloride; MCB, monochlorobenzene; ΣCB, total chlorobenzenes; NH_4^+, ammonium; Eh, redox potential; SO_4^{2-}, sulphate; ΣMn, total manganese.

Interestingly, four clusters could be distinguished by PCA corresponding to geographical zonation: (i) heavily contaminated area (wells A, B, C, D), (ii) SW fringe (G, H, N, O), (iii) SE

fringe (wells E, F, I, L) and (iv) northern fringe of the plume (wells J, M) (Fig. 6-3, Fig. 6-1 for location of the wells). Cluster I representing the high contaminated region of the plume is consequently correlated with high contaminant concentrations. Cluster II grouped positively along PC2, which is associated with manganese, sulfate and $\delta^{13}C_{CB}$ emphasizing relatively higher values for these variables at the SW fringe. Although enrichment in ^{13}C indicating biodegradation has been observed in this region, a linkage between anaerobic oxidation and presence of sulfate or manganese could not be ruled out. Samples from the SE fringe of the plume (cluster III) grouped close to the origin and thus show intermediate hydrogeochemical patterns with no extreme values in the variables taken into account for PCA. Alternatively, sampling locations of the northern fringe (cluster IV) clustered positively along PC1 and displayed relatively higher values of ferrous iron, methane, TOC and electric conductivity. At this area, MCB is present > 60% of total CB and isotope enrichment of CB and MCB is likely to mirror MCB degradation. Because $\delta^{13}C_{MCB}$ values were positively correlated with ferrous iron, TOC and electric conductivity ($P<0.05$), a linkage between these geochemical indicators and degradation activity may exist. Indeed, TOC may serve as a source of electron donors to support RDH of CBs. Moreover, positive correlation of ^{13}C enriched MCB with increased ferrous iron concentrations may suggest anoxic oxidation of MCB during ferric iron reduction.

Caution should be taken classifying sampling locations, as PCA directly reflects the limits of the methodology on which each variable integrated in the analysis has been derived. For instance, samples belonging to cluster I were collected in a highly contaminated part of the plume and showed no shift in $\delta^{13}C$ values of MCB or of total CBs. This could be used as indicator for recalcitrance to biodegradation. However, CSIA suggested DCB degradation due to relatively high isotopic enrichment accompanied by MCB formation (Tab. S4, Fig. S5, Anhang F). The significant DCB enrichment was masked in the isotope balance possibly due to the accumulation of relatively ^{13}C depleted MCB which may point to RDH of DCB. Moreover, the results of the *in situ* and laboratory tracer studies showed that *in situ* biodegradation of MCB is feasible under the ambient aquifer conditions, but could not be determined by means of CSIA. This emphasizes the interest of combining several tools of various sensitivity levels to gain evidence of *in situ* degradation.

Overall, the PCA results underlined the hydrogeochemical heterogeneity of the investigated field site. Delineated clusters of sampling locations showed various hydrogeochemical trends and corresponded to defined zones of the contamination plume, which putatively differ with respects to their potential and development of CB degradation activity. However, causality or mechanistic interpretation could often hardly be proved based on statistical analysis of the field data alone. Together with other lines of evidence, this delineation may be relevant in terms of risk assessment when coupling information about dominant contaminant flow patterns with zonation of potential biogeochemical characteristics at the plume scale.

To further confirm that monitored natural attenuation might be suitable as remediation method at the study site stability of the plume as well as of NA processes needs to be demonstrated (US-EPA, 1999). The comparative analysis of contaminant and isotope signature patterns of sampling locations in year 2005 and 2006 by PCA did not reveal substantial changes (Fig. S6, Anhang F). Additionally, the temporal evolution of contaminant concentration (2000-2007) and corresponding isotope signature (2005-2007) were analyzed in more detail for wells H, F, L at the southern fringe of the plume (Fig S7-9, Anhang F). In all three wells, concentrations varied significantly while the isotope signatures remained relatively stable over time. Although minor fluctuations within the data occurred, the constant enrichment of heavy isotopes compared to central parts of the plume indicated that the biodegradation potential was maintained over time demonstrating plume stability presumably controlled by microbial activity (see also SI, section 2.5; Anhang F).

6.5.5 Implications for environmental studies and site management in terms of natural attenuation

This study aimed at evaluating the efficacy of NA applying an integrated approach. Multivariate statistics permitted to delineate various geochemical zones within the plume associated with microbial degradation suggesting variability of processes involved in contaminant removal underlining the complexity and heterogeneity of the field site. PCA supported the results obtained by the isotope balance and enabled a combined analysis of geochemical and isotope data to evaluate the *in situ* biodegradation potential. To explore significant spatial and temporal changes of the hydrogeochemical patterns statistical methods are helpful and CSIA can reveal if biodegradation controls plume stability. Such methods can be easily implemented into a monitoring campaign. At the investigate area plume stability was demonstrated over time. Major changes in environmental conditions that may reduce the efficacy of the NA processes especially that of *in situ* biodegradation, are not likely to occur at the field site.

As basically required by authorities and scientists we presented an integrative concept encompassing multiple lines of evidence for NA of recalcitrant contaminants such as CBs at contaminated complex subsurface environments (McKelvie et al., 2007; US-EPA, 1999). At many field sites numerous contaminants are present and different mechanisms may govern the fate of pollutants *in situ*. Especially at such complex systems characterized by multiple contaminants, sequential degradation and/or different potential pathways one single method can not elucidate all relevant biogeochemical processes. Therefore, coupling of several techniques may lead to a more robust assessment and allows verifying plausibility of lines of evidence for *in situ* biodegradation processes. This further results in complex sets of data which can be treated more easily by statistical methods to interpret the relationship between certain parameters and to analyze the influence as well as temporal changes of

hydrogeochemical factors on *in situ* biodegradation without substantial additional monitoring costs (Andrade et al., 2008; Lee et al., 2001).

Even for recalcitrant contaminants (e.g. MCB) with unknown degradation pathways, an appropriate methodology may provide evidence of *in situ* biodegradation. An integrative approach consisting of hydrogeochemical analysis combined with novel stable isotope tools and multivariate statistics may substantially support identification of mechanisms and main biogeochemical factors associated with contaminant removal. At the complex chlorobenzene contaminated field site evaluation of NA potential showed that CBs are subjected to microbial degradation leading to sustainable removal of pollutants from the groundwater.

6.5.6 Acknowledgment

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6.5.7 Supporting Information Available

Detailed description of the field site, sampling, experimental set up, analytical methods, hydrogeochemical data, chromatograms of *in situ* microcosm analysis, $\delta^{13}C_{CO2}$ of laboratory enrichment cultures, isotope analysis of chlorinated benzenes, plume stability is available free of charge via the Internet at http://pubs.acs.org (Anhang F).

7 Assessment of *in situ* biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland

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7.1 Abstract

The degradation of monochlorobenzene (MCB) was assessed in a constructed wetland treating MCB contaminated groundwater using a detailed geochemical characterisation, stable isotope composition analysis and *in situ* microcosm experiments. A correlation between ferrous iron mobilisation, decreasing MCB concentration and enrichment in carbon isotope composition was visible at increasing distance from the inflow point, indicating biodegradation of MCB in the wetland. Additionally, *in situ* microcosm systems loaded with ^{13}C-labelled MCB were deployed for the first time in sediments to investigate the biotransformation of MCB. Incorporation of ^{13}C-labelled carbon derived from the MCB into bacterial fatty acids substantiated *in situ* degradation of MCB. The detection of ^{13}C-labelled benzene indicated reductive dehalogenation of MCB. This integrated approach indicated the natural attenuation of the MCB in a wetland system. Further investigations are required to document and optimise the *in situ* biodegradation of MCB in constructed and natural wetland systems treating contaminated groundwater.

7.2 Capsule

An integrated approach including isotope composition analysis and *in situ* microcosm experiments provided evidences for *in situ* biodegradation of MCB in a wetland system.

7.3 Introduction

Monochlorobenzene (MCB) is encountered worldwide as a groundwater pollutant, and persists in the essentially anaerobic aquifer at the large-scale contaminated site in Bitterfeld, Germany (Heidrich et al., 2004b; Wycisk et al., 2004). In recent years, interest has grown in using phytoremediation processes for the elimination of recalcitrant organic substances from waste- and groundwater (Macek et al., 1998; Schnoor et al., 1995; Shimp et al., 1993; Trapp, 2000) including chloroaromatics (Gilbert and Crowley, 1997). Wetland systems represent an effective and inexpensive option to treat groundwater polluted with organic compounds by taking advantage of the geochemical and biological processes (e.g. Baker, 1998; Dunbabin and Bowmer, 1992; Gumbricht, 1993). Indeed, rapid degradation of chlorinated organics has been observed in the rhizosphere (Anderson and Walton, 1995; Jordahl et al., 1997; Lorah and Olsen, 1999; Pardue et al., 1996).

While aerobic degradation of MCB has been well studied (Van Agteren et al., 1998), only some evidence for MCB transformation under anoxic conditions has been presented yet and the degradation pathway is unknown (Kaschl et al., 2005; Liang and Gribic-Galic, 1990; Nowak et al., 1996). Moreover, only very few studies focus on the anaerobic microbial transformation of MCB under field conditions. Recently, indications of anaerobic MCB degradation taking place in the Bitterfeld contaminated aquifer were provided on the basis of isotope fractionation patterns (Kaschl et al., 2005). Kinetic isotope fractionation processes have been employed to demonstrate the biological transformation of various contaminants (Lollar et al., 2001; Richnow et al., 2003a; Richnow et al., 2003b; Song et al., 2002). A substantial enrichment of ^{13}C in the non degraded fraction in the course of a contaminant plume indicates microbial degradation, as dilution and sorption do not affect the isotope composition of contaminants significantly (Harrington et al., 1999; Schüth et al., 2003; Slater et al., 2000). Combining stable isotope composition analysis with information obtained in simple *in situ* microcosm experiments (BACTRAPs) using isotope labelled substrate may provide a suitable approach to qualitatively support *in situ* biotransformation and to monitor spatial and temporal natural attenuation processes. Previously, BACTRAPs were exclusively installed in groundwater monitoring wells (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006a) and were deployed in sediment for the first time in the framework of this study.

For the assessment of *in situ* biodegradation in constructed wetlands and wetlands treating contaminated groundwater, it may be necessary to use several methods providing more than one line of evidence. A combined approach may be of additional benefit in particular when systems are complex, possess several compartments and convincing evidence is required. Moreover, a better understanding of the controlling geochemical processes in wetland systems is necessary to reliably predict the retention and transformation of contaminant. In this study, we evaluated the natural attenuation of MCB in a constructed wetland treating MCB contaminated groundwater using a detailed geochemical characterisation, stable isotope composition analysis and *in situ* microcosm experiments. The spatial variations of geochemical parameter were studied with the help of multivariate statistics to investigate the main processes controlling the wetland system. The concentration and carbon stable isotope composition of MCB was analysed to monitor the *in situ* contaminant degradation and *in situ* microcosms were used to provide qualitative evidences of *in situ* biotransformation of MCB.

7.4 Materials and methods

7.4.1 Design and characteristics of the wetland

The pilot constructed wetland at the experimental site in Bitterfeld was set up in December 2002. The horizontal subsurface flow wetland consisted of a stainless steel tank divided into

two segments. Each segment was 6 m x 1 m and was filled to an average depth of 0.5 m with autochthonous quaternary aquifer material consisting predominantly of Bitterfeld mica sand (25%) and gravel (67%), which was embedded in lignite (10%) with an effective porosity of 28% (Vogt et al., 2002). The hydrogeochemical characteristics of the study site and the filling material originating from the local aquifer are described in previous studies (e.g. Vogt et al., 2002; Weiss et al., 2001). One of the segments was planted with common reed (*Phragmites australis*, Cav.), whereas the other side was left unplanted. In both segments, a 1 m long open water pond at the outflow side allows direct contact between the atmosphere and the water surface. The water level was maintained at approximately 10 cm below the surface of the wetland. The groundwater was collected from the MCB contaminated aquifer and conveyed from 16 to 22 m depth directly to the wetland. Both segments were operated in a flow-through mode at a flow rate of 4.7 L h^{-1}, corresponding to a retention time of 6 days.

7.4.2 Sampling

In the period from April to September 2005, pore water samples were collected five times (d0; d53; d66; d143; d172) in order to investigate the geochemical processes and the contaminant behaviour in the wetland system. The pore water was collected in both segments along a transect from the inflow up to the outflow of the wetland, at respectively 0 (inflow valves), 0.5, 1, 2, 3 and 4 m using a stainless steel lance. At each sampling point, three depths, 30, 40 and 50 cm were systematically investigated. Water samples were also collected at the ponds (6 m).
In addition, to assess the *in situ* biodegradation using isotope composition analysis, pore water samples from both segments were collected at day 0 and day 53, at 0, 1 and 3.5 m along the wetland at 0.5 m depth, as well as in the ponds.

7.4.3 Physico-chemical and geochemical parameters of the pore water samples

The redox potential was measured on-line in the field using a SenTix ORP electrode (PT 1000, PreSens, Regensburg, Germany). The temperature was determined by a temperature sensor (PT 1000, PreSens, Regensburg, Germany). Samples for the pH analysis and the quantitative ions were filtered through a 5 µm syringe filter (Ministart NML, Sartorius) for particle removal. The pH value was measured with a SenTix41 electrode with pH 537 Microprocessor (WTW, Weilheim, Germany). Oxygen measurement was carried out using an optical oxygen trace sensor system (oxygen meter Fibox-3-trace and flow-through cell type sensor FTC-TOS7) with automatic temperature compensation (temperature sensor PT 1000) (PreSens, Regensburg, Germany). For the analysis of Mn(II), total iron and Fe(II), hydrochloric acid was added and samples were diluted with deionised water (1:10, v:v). Total iron and Mn(II) concentrations were analysed by atomic emission spectrometry with ICP excitation and CCD detection (Spectro Ciros Vision CCD, Spectro Analytical Instruments, Kleve, Germany). Photometric

analysis of ferric iron was carried out at 562 nm after derivatisation with ferrocin using a Cadas 100 photometer (Hach Lange, Düsseldorf, Germany). Chloride and sulphate concentrations were determined by ion chromatography (DX 500) with conductivity detection (CD 20) and a IonPacAG11 (4x250 mm) column (Dionex Corporation, Sunnyvale, USA). For the analysis of sulphide concentrations samples were spiked with sulphide anti-oxidant buffer (200 ml L^{-1} 10 M NaOH, 35 g L^{-1} ascorbic acid, 67 g L^{-1} EDTA) (1:1, v:v) and measured with an ion selective Ag/S 500 electrode and reference electrode R 503 (WTW, Weilheim, Germany).

7.4.4 Analysis of benzene, MCB and metabolites

Pore water samples for the analysis of benzene and MCB concentrations were collected in 20 mL glass flasks (Supelco, Bellefonte, USA), and sealed with Teflon-lined septa. Sodium azide solution was added to the samples to inhibit microbial activity. Benzene and MCB concentrations were quantified by automatic headspace gas chromatography using an HP 6890 gas chromatograph with flame ionisation detector (Agilent technologies, Palo Alto, USA). For headspace analysis a volume of 1000 µl was injected at an injection temperature of 250°C with split 1:5 (measurements in duplicates). The chromatographic separation was achieved on a HP-1 capillary column (Agilent technologies, Palo Alto, USA) (30 m x 0.32 mm x 5 µm) with the following oven temperature program: 45°C (1 min), 20°C min^{-1} to 200°C (2.5 min), 65°C min^{-1} to 250°C (1 min) and a detector temperature of 280ºC.

For the determination of the carbon isotope composition of MCB, 1 L glass bottles (Schott, Mainz, Germany) containing NaOH pellets to prevent microbial growth were filled completely with groundwater, stored at 4°C and extracted within 24 h using 2 mL n-pentane as described previously (Richnow et al., 2003b). The analysis of volatile metabolites obtained in the *in situ* microcosm experiments was carried out using a HP 6890 gas chromatograph with HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, USA). Aliquots of 1 µl liquid samples were injected at a temperature of 280°C with split 1:40 and separated on a Zebron BPX-5 column (30 m x 0,32 mm x 0,25 µm) (Phenomenex, Torrance, USA). The following oven temperature program was applied: 40°C (2.5 min), 10°C min^{-1} to 70°C (0 min), 60°C min^{-1} to 280°C (4 min).

7.4.5 *In situ* microcosms (BACTRAPs)

Preparation of microcosms and derivatisation of fatty acids

The *in situ* microcosms were prepared as described previously (Stelzer et al., 2006a). Different sets of *in situ* microcosm experiments were prepared. One set was loaded with [$^{13}C_6$]-labelled MCB (Cambridge Isotope Laboratories, Andover, USA) and another one with natural abundance MCB. A third set was kept unloaded to observe the background effects. The loading was done via gas phase under reduced pressure with approximately 40 mg MCB per g Bio-Sep®. The microcosms were deployed at 1.5, 2.5 and 4.5 m from the inflow in both planted

and unplanted segments at 50 cm depth. The microcosms were collected after 6 weeks and fatty acid extraction was carried out according to Bligh and Dyer (1959). The derivatisation to obtain fatty acid methyl esters (FAME) was done according to Thiel et al. (2001). After evaporation to complete dryness and addition of heneicosanoic acid methyl ester (C21:0) as an internal standard the FAME fraction was dissolved in *n*-hexane for further identification, structural characterisation and carbon isotope composition analysis.

GC-MS analysis

For identification and structural characterisation of FAME a HP 6890 gas chromatograph coupled with a HP 5973 quadrupol mass spectrometer (Agilent Technologies, Palo Alto, USA) was used. The FAMEs were separated on a Zebron BPX-5 column (30 m x 0.32 mm x 0.25 µm) (Phenomenex, Torrance, USA) with the following temperature program: 70°C (1 min), 20°C min^{-1} to 130°C, 2°C min^{-1} to 150°C (5 min), 2°C min^{-1} to 165°C (5 min), 2°C min^{-1} to 230°C, 20°C min^{-1} to 300°C (5 min). FAME were identified by comparing with the retention time and mass spectra of an authentic standard mix (bacterial acid methyl esters mix, Sigma-Aldrich, Germany) and quantified relatively to the internal standard.

Isotopic composition analysis

The carbon isotope composition of MCB and the FAMEs was measured with a gas chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) consisting of a GC unit (HP 6890, Agilent technologies, Palo Alto, USA), a combustion device (Finnigan MAT GC III, ThermoFinnigan Bremen Germany) with water-removal assembly (Nafion® membrane, 50 cm long, T = 0°C) and a mass spectrometer (Finnigan MAT 252; ThermoFinnigan, Bremen, Germany), as previously described (Richnow et al., 2003a). Helium was used as carrier gas at a flow rate of 1.5 ml min^{-1}.

Stable isotope samples were measured in triplicates and the analyses were carried out immediately after each sampling. Aliquots of 1 µl liquid samples were injected at 250°C with split 1:10 to the GC-C-IRMS and separated on a capillary column (Zebron ZB-1, 60 m x 0.32 m x 1 µm; Phenomenex, Torrance, USA). The following chromatographic conditions were applied: injector temperature 250°C, oven temperature program: 40°C (1 min), 4°C min^{-1} to 150°C, 20°C min^{-1} to 250°C (2 min). The carbon isotope composition is reported in the delta notation as $\delta^{13}C$ values [‰] relative to Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) (Eq. 7-1) (Hoefs, 1997).

(Equation 7-1)
$$\delta^{13}C[‰] = \left(\frac{\left(\frac{^{13}C}{^{12}C} \right)_{Sample}}{\left(\frac{^{13}C}{^{12}C} \right)_{Standard}} - 1 \right) * 1000 = \left(\frac{R_{Sample}}{R_{Standard}} - 1 \right) * 1000$$

Eq.(7-2) is applied to calculate the remaining substrate fraction (f_t) using the isotope fractionation factor (αC).

(Equation 7-2) $$f_t = \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\frac{1}{\alpha C}-1}\right)}$$

The R_t and R_0 give the isotope composition of MCB at time t and zero. Eq. (7-3) is applied to calculate the percentage of biodegradation of the residual substrate fraction (B_t).

(Equation 7-3) $$B[\%] = (1 - f_t) * 100$$

For the separation of the FAME fractions, a Restek RTX 5 column (60 m x 0.32 mm x 0.1 µm; Restek, Bellefonte, USA) was used with the same temperature program applied for the GC-MS-analysis of FAME. Aliquots of 1 µl were injected with split 1:5. The methylation of fatty acids for gas chromatographic analysis introduces an additional carbon atom into the structure of the fatty acid molecules which affects its isotopic composition. Therefore the isotope signature of fatty acids ($\delta^{13}C_{FA}$) was corrected for the isotope effect upon derivatisation to FAME with methanol as described previously (Abraham et al., 1998; Abrajano et al., 1994; Goodmann and Brenna, 1992). The methanol used for the derivatisation had an isotope composition of -38.2‰.

7.4.6 Statistical analysis

Statistical analyses were carried out using the R software (R, Version 2.1.1, 2005). Statistical significance of the difference in geochemical parameters as well as concentrations and isotopic compositions of MCB between the planted and unplanted segment was determined with the unpaired Wilcoxon or the Kruskall-Wallis rank sum tests. Correlation analyses were carried out using the Spearman rank sum coefficient. Principal Component Analysis (PCA) was used to analyse the relationship between the different samples with reference to their respective pore water parameters. The sampling location corresponds to the object and the chemical parameters to the descriptors (represented by the vectors) of the multivariate analysis. The PCA were scaled as correlation biplots.

7.5 Results

7.5.1 Characterisation of pore water chemistry

Distribution of MCB and benzene

The MCB concentration was measured as a function of the distance from the inflow point in both the planted and unplanted segment (Tab. 7-1). The average amount of MCB ranged from

14.4 mg L^{-1} to 17.7 mg L^{-1} at the inflow down to 2.0 mg L^{-1} to 2.2 mg L^{-1} in the ponds for the planted and the unplanted segment, respectively. No significant difference in MCB concentration between the three depths over the study period were generally observed ($p<0.05$). Benzene was found in low concentration in both segments (<26 µg L^{-1}), with generally higher concentration values in the unplanted segment (Tab. 7-1).

Tab. 7-1: Geochemical characterisation, MCB and benzene concentrations of samples collected from (a) unplanted plot and (b) planted plot. Values represent the depth profiles average in the soil compartments (0.5 - 4 m) and average (0 m and pond) over the study period (13.04.05-29.09.05). Standard deviation is indicated in parentheses.

Sampling point [m from inflow]	pH	Temp. [ºC]	O_2 [mg L^{-1}]	Eh [mV]	Fe(II) [mg L^{-1}]	Sulphate [mg L^{-1}]	Cl^- [mg L^{-1}]	Benzene [µg L^{-1}]	MCB [mg L^{-1}]
(a)									
0	7.1 (3.6)[d]	14.8 (8.5)[d]	0.04 (0.02)[e]	38 (37)[d]	0.18 (0.23)[c]	969 (402)[c]	280 (116)[c]	25.6 (3.6)[c]	17.7 (8.0)[c]
0.5	7.0 (2.2)[b]	16.3 (5.4)[a]	0.05 (0.04)[b]	61 (26)[b]	2.43 (1.83)[a]	969 (253)[a]	273 (69)[a]	21.2 (6.1)[a]	14.6 (5.0)[a]
1	6.7 (2.1)[a]	17.1 (5.4)[a]	0.04 (0.02)[a]	84 (38)[a]	9.58 (5.80)[a]	967 (253)[a]	269 (68)[a]	23.3 (3.9)[a]	14.5 (5.1)[a]
2	6.9 (2.0)[a]	17.8 (5.5)[a]	0.04 (0.04)[a]	70 (41)[a]	12.40 (6.74)[a]	973 (254)[a]	267 (67)[a]	22.2 (5.4)[a]	14.5 (5.2)[a]
3	6.8 (1.9)[a]	18.4 (5.5)[a]	0.04 (0.03)[a]	57 (34)[a]	21.12 (7.45)[a]	974 (254)[a]	261 (71)[a]	22.5 (5.5)[a]	14.9 (5.6)[a]
4	6.8 (1.9)[a]	18.9 (5.6)[a]	0.04 (0.02)[a]	57 (37)[a]	30.23 (7.08)[a]	973 (256)[a]	267 (72)[a]	21.1 (6.3)[a]	14.4 (5.5)[a]
Pond	6.8 (3.0)[d]	20.9 (8.4)[d]	n.a.	250 (44)[d]	3.06 (8.81)[c]	986 (403)[c]	287 (118)[c]	1.8 (3.0)[c]	2.0 (7.9)[c]
(b)									
0	6.8 (3.1)[d]	14.8 (8.5)[d]	0.04 (0.02)[e]	35 (40)[d]	0.20 (0.21)[c]	977 (400)[c]	262 (109)[c]	24.0 (6.9)[c]	14.4 (6.4)[c]
0.5	6.8 (2.2)[b]	15.6 (5.5)[a]	0.13 (0.05)[b]	47 (42)[b]	5.73 (3.62)[a]	994 (251)[a]	257 (68)[a]	11.8 (5.5)[a]	8.7 (7.7)[a]
1	6.8 (2.1)[a]	16.6 (5.4)[a]	0.12 (0.09)[a]	54 (40)[a]	13.34 (7.48)[a]	980 (248)[a]	256 (68)[a]	15.7 (6.6)[a]	9.8 (7.2)[a]
2	6.7 (2.0)[a]	17.6 (5.4)[a]	0.05 (0.02)[a]	46 (36)[a]	25.48 (7.60)[a]	1006 (248)[a]	266 (68)[a]	11.9 (8.8)[a]	8.2 (6.6)[a]
3	6.7 (1.9)[a]	18.0 (5.4)[a]	0.05 (0.03)[a]	51 (34)[a]	36.57 (7.33)[a]	1000 (248)[a]	271 (69)[a]	11.3 (8.9)[a]	7.9 (6.1)[a]
4	6.5 (1.9)[a]	18.8 (5.5)[a]	0.03 (0.02)[a]	57 (34)[a]	37.40 (8.94)[a]	998 (259)[a]	266 (74)[a]	10.2 (8.0)[a]	7.7 (5.9)[a]
Pond	6.8 (3.1)[d]	20.5 (8.1)[d]	4.85 (1.89)[e]	204 (43)[d]	2.42 (9.28)[c]	1068 (397)[c]	275 (111)[c]	0.2 (0.4)[c]	2.2 (7.8)[c]

[a] Profile average of five sampling dates (n=15)

[b] Profile average of four sampling dates (n=12)

[c] Average of five sampling dates (n=5)

[d] Average of four sampling dates (n=4)

[e] Average of three sampling dates (n=3)

n.a. not assessed

Pore water geochemistry

The evolution of oxygen, redox potential, manganese, sulphide, sulphate, ferric iron and total iron were monitored along the flow path at three depths in order to characterise the geochemical conditions prevailing in the wetland system. The average values of the three depths investigated at each sampling point (0, 0.5, 1, 2, 3, 4 m from the inflow) were computed (Tab. 7-1). To investigate the differences between the sampling locations and to explore existing gradients, the data sets were analysed by principle component analysis, separately for the unplanted and the planted segments (Fig. 7-1). In both cases, the vectors representing the temperature, Fe(II), and total Fe parameters were orientated in the same direction (positive correlation), in the opposite direction with regards to vectors representing manganese and

ammonium (negative correlation), and are perpendicular to the MCB vector (absence of correlation). The orthogonal projection of an object on a descriptor allows approximating the correlation between that object and the descriptor. For both segments, samples from the inflow part of the wetland (0.5 to 1 m) were associated with MCB, manganese and ammonium, whereas samples from the outflow (3 and 4 m) were generally associated with total Fe and Fe(II). Indeed, the Fe(II) concentration at the inflow were below 0.5 mg L^{-1} and systematically increased along the flow path in both planted and unplanted segment, indicating ferrous iron mobilisation. A clear shift in the parameters characterising predominantly the trends of variation of the samples along the flow path is operating on the first principal component. Indeed, the axis I corresponds to a spatial gradient from the inflow to the outflow of the system, and separates sampling sites accordingly (from the right to the left). The variations of geochemistry along the flow path contributed more in characterising the samples than the variations occurring along the vertical profile.

The maximum concentration of Fe(II) reached an average value of 37.4 mg L^{-1} at 4 m from the inflow of the planted segment. The levels of total dissolved iron detected throughout the experimental period ranged from 0.8 to 50.6 mg L^{-1}. Interestingly, Fe(II) mobilisation was more important in the planted than in the unplanted segment. Correlation analysis revealed, however, very similar Fe(II) mobilisation patterns in both segments over the study period ($\rho>0.9$; $p<0.01$). Iron reduction was apparently a relevant process at the three depths. Due to the high background concentration of sulphate in the supplied groundwater (700-1100 mg L^{-1}), a sensitive analysis of the microbial sulphate reduction on the basis of sulphate concentration was not possible. However, trace concentrations of sulphide (<3 µg L^{-1}) were detected along the horizontal transect, indicating sulphate reduction. Mn(II) concentrations systematically ranged below 0.2 mg L^{-1}, suggesting a low relevance of Mn as electron acceptor in the wetland (data not shown). The redox potential displayed averages of values ranging from 35 mV at the inflow to 250 mV in the ponds, and maximal concentration of oxygen in the soil compartments was 0.12 mg L^{-1}, indicating the prevalence of anoxic conditions in the soil compartments and oxic conditions in the ponds, respectively. The dissolved organic and total carbon concentrations ranged from 0.24 to 0.45 mM L^{-1} and from 2.27 to 2.41 mM L^{-1} in the unplanted and planted segments, respectively (data not shown). Nitrate concentrations were assessed in previous studies and ranged systematically under the detection threshold in the groundwater supplied to the wetland (Vogt et al., 2002), and nitrate may therefore not represent a relevant electron acceptor. The chloride concentrations did not show any significant variation along the transect.

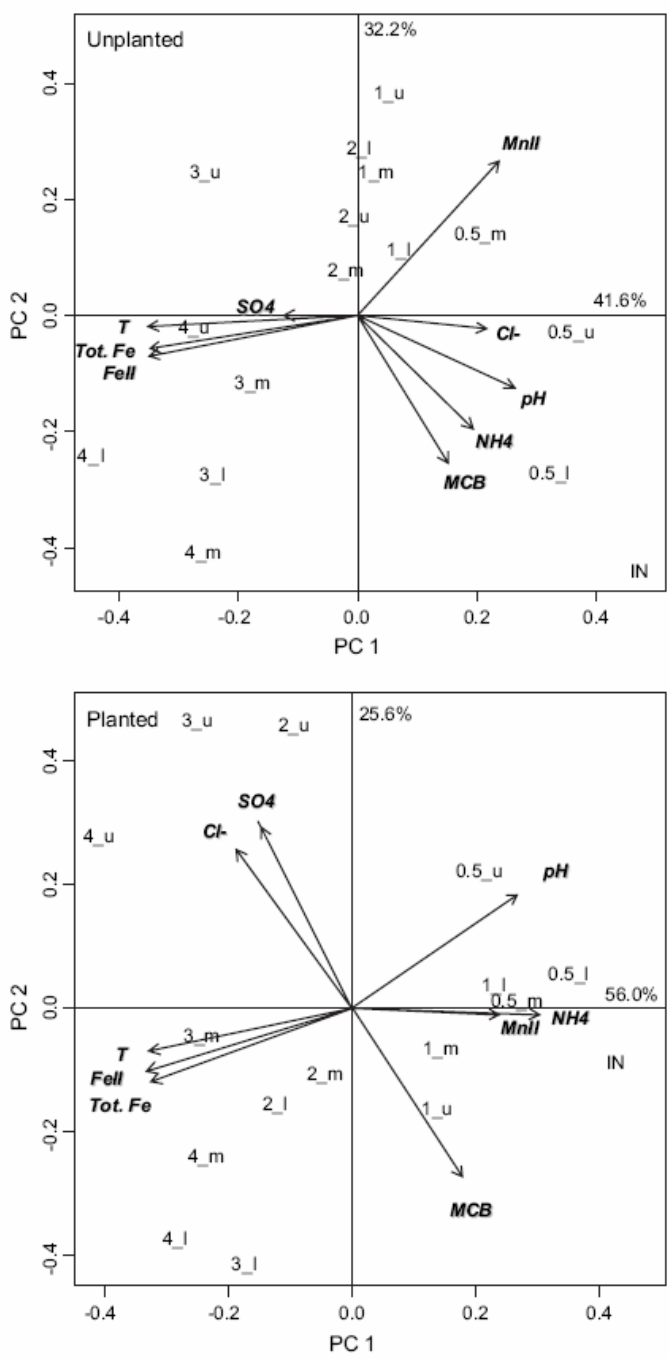

Fig. 7-1: Ordination plot generated by principal component analysis representing the relationship between the sampling locations and the average geochemical parameters measured in both the planted (a) and the unplanted (b) experimental wetlands over the study period. Description vectors correspond to: FeII = ferric iron; Tot.Fe = Total iron (Fe(II)+Fe(III)); MCB = Monochlorobenzene; MnII = Manganese(II); NH4. = Ammonium; T = Temperature. Objects correspond to: 0.5 to 4 = distance [m] from the inflow point; u = upper depth (30cm); m = medium depth (40cm); l = lower depth (50cm). Values on the axes indicate % of total variation explained by the axes. PC 1 = principal component axis 1; PC 2 = principal component axis 2.

7.5.2 Carbon isotopic composition of MCB

The concentration and isotopic composition ($\delta^{13}C$) of MCB were plotted as a function of the distance from the inflow (Fig. 7-2). A decrease in MCB concentrations over the flow path was

systematically associated in both segments with a significant enrichment in $\delta^{13}C$. In the planted segment, MCB showed a maximal isotope shift of 0.6‰, whereas it reached 0.9‰ in the unplanted segment. This indicates that MCB is subjected to *in situ* biodegradation in both segments. Assuming an isotopically homogenous source of MCB, these values are slightly higher than the typically defined analytical error of 0.5‰ associated with compound specific isotope analysis (Dempster et al., 1997; Mancini et al., 2002).

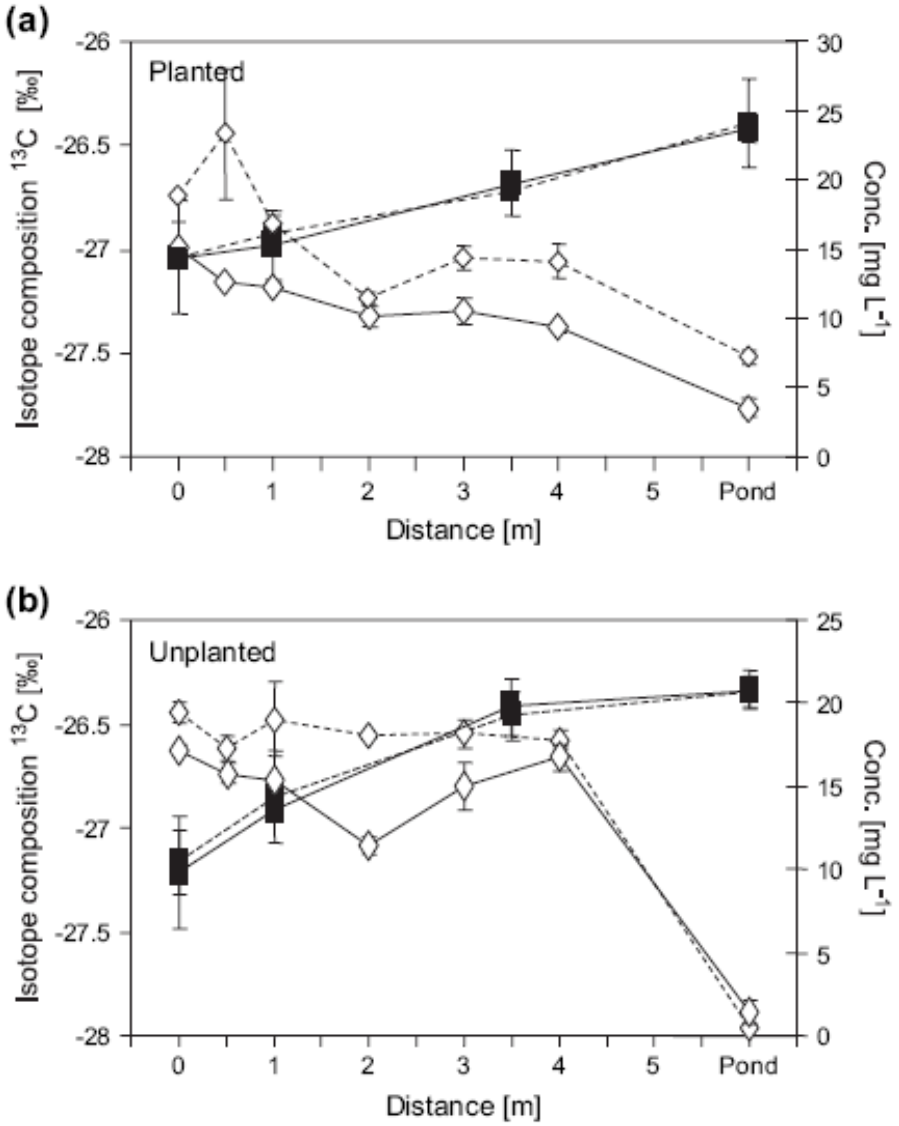

Fig. 7-2: Concentration and isotopic composition of MCB (diamond = concentration; squares = isotopic composition) in both planted (a) and unplanted (b) segments and for both sampling dates (line = 14.04.2005, dashed = 09.05.2005). Error bars show the standard deviation.

A quantitative assessment of *in situ* MCB biodegradation requires a fractionation factor representing the *in situ* conditions. Fractionation factors for aerobic MCB degradation are available (Kaschl et al., 2005), whereas factors retrieved under laboratory conditions for anaerobic MCB degradation are missing. Although MCB degradation under anaerobic conditions is expected in the wetland, Eq. (7-3) allows estimating the significance of

degradation along the flow path throughout the wetland applying the highest isotope fractionation factors (αC) retrieved for aerobic MCB degradation by a dioxygenase reaction pathway (αC = 1.0004) (Kaschl et al., 2005). The quantification was carried out based on the isotope signature of MCB measured at the planted side at day 0. The lowest $\delta^{13}C$ value measured (inflow point, 0 m: MCB $\delta^{13}C$= -27.0 ‰) was used as the initial isotope composition of the source (R_0). The estimated percentage of biodegradation was 60% of the inflowing MCB mass at 3.5 m, whereas the observed contaminant mass decrease reached only 38% at 4 m from the inflow. Processing of isotope and concentration data of the second sampling campaign gave almost identical results.

The use of too low fractionation factors for the quantification of MCB degradation would lead to an overestimation of the effective MCB mass depletion at the planted segment. The observed absence of significant MCB mass depletions along with a higher isotopic shift at the unplanted segment obviously direct to the same conclusion. Therefore, higher fractionation factors are expected, which would be more in concordance with the observed MCB concentration values. The use of higher isotope fractionation factors would point to a dominating anaerobic fractionation process, which is compatible with the observation of anoxic conditions in the wetland. The fractionation factors would then be comparable to the ones retrieved by Kaschl et al. 2005 (Kaschl et al., 2005) (αC = 1.0005) in the local anaerobic aquifer with MCB contamination or by Griebler et al. (2004a) and Mancini et al. (2003), obtaining significantly higher fractionation factors for the anaerobic degradation of benzene or trichlorobenzenes.

7.5.3 *In situ* microcosm experiment

[$^{13}C_6$] labelled MCB was used as stable isotope tracer in the *in situ* microcosm experiment in the wetland. The BACTRAPs were incubated directly in both soil compartments, at several distances from the inflow, as well as in the ponds. The incorporation of labelled carbon derived from the labelled substrate into bacterial fatty acids provided evidence for MCB degradation in the wetland system. In addition, [$^{13}C_6$] benzene, a possible intermediate, found on the BACTRAPs from both segments demonstrated the occurrence of reductive dehalogenation of MCB (Fig. 7-3). Even though the employed [$^{13}C_6$] MCB contained 0.02 % of [$^{13}C_6$] benzene as impurity, significantly higher amounts of [$^{13}C_6$] benzene were detected by GC-MS.

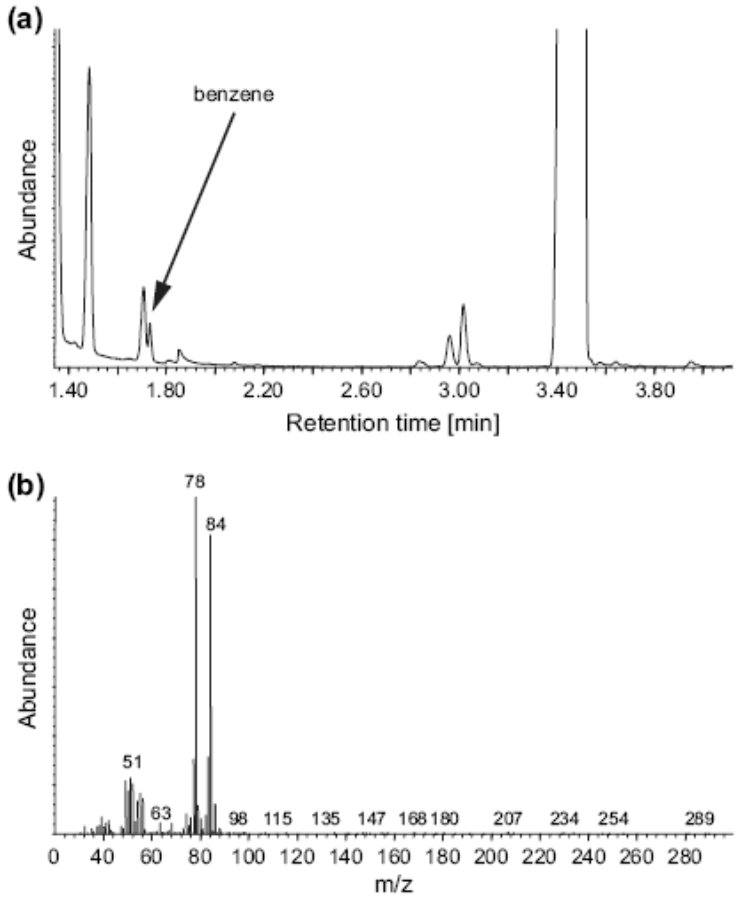

Fig. 7-3: GC-MS chromatogram (total ion current) of the *in situ* microcosm extract showing the ^{13}C-labelled benzene metabolite at retention time of 1.73 min and corresponding mass spectrum (a) with the molecule mass of m/z = 84 for [$^{13}C_6$] benzene and m/z 78 for [^{12}C] benzene (b).

Fatty acid composition

The composition of total fatty acid fractions extracted from *in situ* microcosms was compared to investigate variation in the microbial community. No systematic differences were observed between the oxic ponds and the anoxic segments of the wetland. However, the lowest quantity of fatty acids was retrieved from the microcosms exposed at 4.5 m in the soil compartments, and the highest accumulated biomass was retrieved from the microcosms deployed in the pond of the unplanted side of the wetland (Fig. 7-4).

The fatty acid patterns were dominated by high amounts of the saturated hexa- (C16:0) and octadecanoic (18:0) and the monounsaturated hexa- (C16:1) and octadecenoic (C18:1) acids which are common fatty acids in bacteria. The tetra- (C14:0), pentadecanoic (C15:0) and eicosanoic (C20:0) acids, the *iso* and *anteiso* isomers of C15:0 as well as the unsaturated C18:2 were present in lower abundance and could not be detected in all samples (Fig. 7-4).

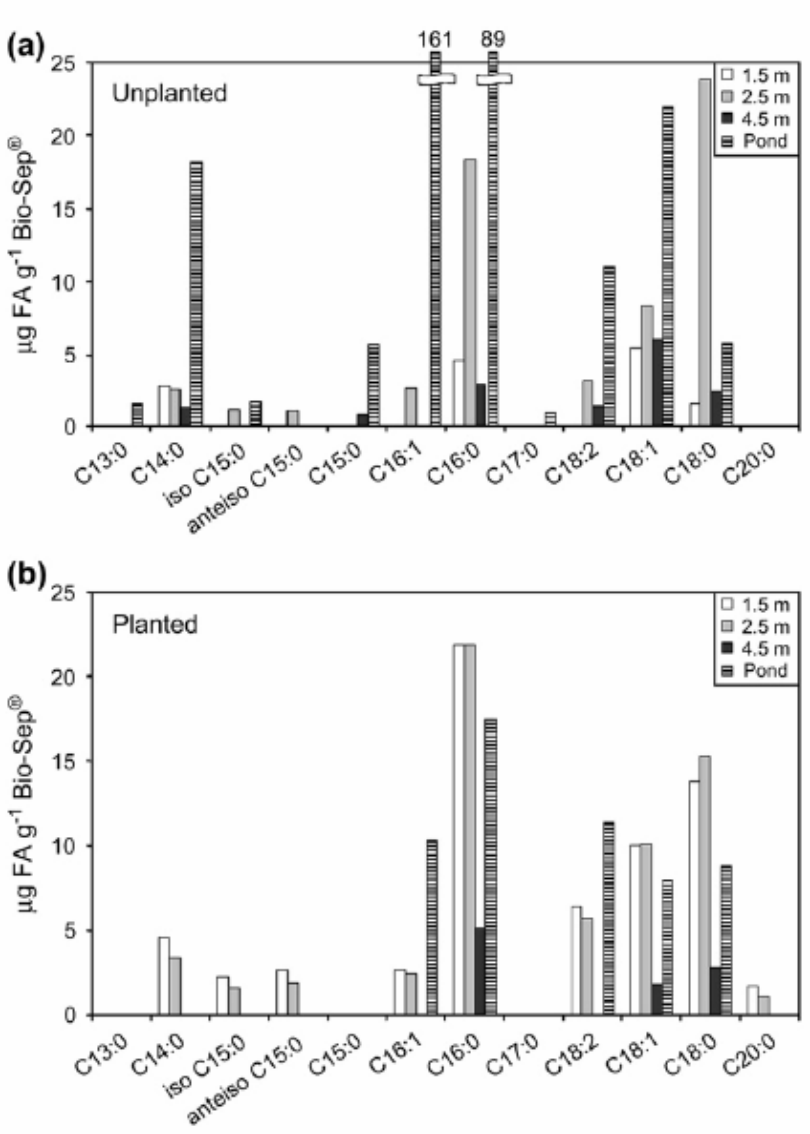

Fig. 7-4: Absolute abundance of extracted fatty acids [µg FA per gram Bio-Sep® beads] from *in situ* microcosms exposed in the soil compartment at different distance from the inflow and ponds at the unplanted (a) and planted (b) segments of the constructed wetland.

The variation of the geochemical parameters down gradient of the inflow were not related to a distinct change in fatty acid composition in both segments. No systematic differences in the fatty acid patterns were found between the planted and unplanted segments. Globally, no clear indications of microbial community changes were obtained on the basis of the fatty acid composition. In this experiment, fatty acid patterns were probably not sensitive enough to reflect changes in the microbial communities as a function of variation in geochemical conditions within the constructed wetland.

Isotope signatures of fatty acids

The total lipid fatty acids extracted from the *in situ* microcosms displayed some differences in the incorporation of ^{13}C into fatty acids. This was particularly obvious when comparing the

samples from the soil compartments and the ponds. The isotope composition of fatty acids ($\delta^{13}C_{FA}$) extracted from the BACTRAPs amended with [$^{13}C_6$] MCB ranged between -39‰ and 7244‰ (Tab. 7-2). An enriched ^{13}C signature of fatty acids (> 0 ‰) can only stem from the microbial utilisation of [$^{13}C_6$] MCB as a carbon source. In contrast, fatty acids with an isotope signature lower than -20 ‰ showed the typical natural abundance of $\delta^{13}C_{FA}$ found in soil and aquifer material (Pelz et al., 2001b; Pombo et al., 2002). Fatty acids derived from parallel microcosms with non labelled MCB or from non amended *in situ* microcosms displayed an isotope signature ranging from -24‰ to -54‰ (data not shown). This represents a typical isotope composition of lipids derived from microorganisms feeding on organic substrates with natural isotope composition.

Tab. 7-2: Carbon isotope composition of fatty acids extracted from *in situ* microcosms incubated for 6 weeks with $^{13}C_6$ labelled monochlorobenzene at 1.5, 2.5 and 4.5 m from the inflow point as well as at the pond.

	Planted				Unplanted			
	$\delta^{13}C_{FA}$ [‰]				$\delta^{13}C_{FA}$ [‰]			
[m] from inflow	1.5	2.5	4.5	Pond	1.5	2.5	4.5	Pond
C13:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	118
C14:0	-6	40	n.d.	n.d.	138	204	116	238
iC15:0	121	89	n.d.	n.d.	n.d.	742	n.d.	1597
aC15:0	23	68	n.d.	n.d.	n.d.	3	n.d.	n.d.
C15:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-37	252
C16:1	411	453	n.d.	7244	n.d.	1832	n.d.	806
C16:0	2	9	93	1711	260	95	192	639
C17:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	242
C18:2	-31	-30	n.d.	65	n.d.	-27	-30	216
C18:1	-30	-29	-28	276	-23	-23	-24	175
C18:0	-27	-28	-24	449	-22	-27	-26	266
C20:0	-37	-39	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. – not detected

Generally, all fatty acids extracted from the *in situ* microcosms exposed in the ponds showed enrichment in ^{13}C (65‰ to 7244‰), which was generally of higher intensity than in the soil samples (-39‰ to 1832‰). In contrast, only fatty acids with up to 16 carbon atoms showed ^{13}C incorporation in the soil samples with highest enrichment in C16 species. Fatty acids with longer carbon chains such as C18:0 or C18:1 displayed no enrichment in ^{13}C (-22‰ to -39‰), indicating that the microbial community was not exclusively growing on the [$^{13}C_6$] MCB. Comparing the samples from the planted and unplanted segments no significant differences in

$\delta^{13}C_{FA}$ were observed. Therefore, the putative impact of plants on the microbial community involved in MCB degradation could not be assessed using our test system.

Some fatty acids can be used as biomarker to identify specific groups of microorganisms (Kaur et al., 2005; Zelles, 1999). In some of the samples labelled *iso* and *anteiso* branched fatty acids with 15 carbon atoms could be identified, indicating that Gram-positive bacteria were involved in the biodegradation of MCB. Labelled C18:2 was only found in samples of the ponds. Linoleic acids (C18:2) can serve as a biomarker for fungi or other eukaryotic organisms (Lösel, 1988) and their presence may lead to the hypothesis that grazing organisms, not involved in the biodegradation of MCB, may feed on the microbial biofilm. However, the applied GC-MS procedure did not allow conclusive identification of the position of the double bond.

7.6 Discussion

The geochemical parameters indicated the overall prevalence of anoxic conditions associated with iron mobilisation in the soil parts of the wetland, whereas an aerobic milieu characterised the ponds. In the *in situ* microcosm experiments, the level of incorporation of labelled carbon into bacterial biomass was used as direct indicator of *in situ* MCB degradation. Interestingly, the analysis of the BACTRAPs incubated in the ponds revealed fatty acids patterns and ^{13}C incorporation levels differing from the ones retrieved from the soil compartments. The higher ^{13}C incorporation level observed in the ponds of both segments is indicative of a more effective microbial transformation of the [$^{13}C_6$] MCB under the prevailing aerobic conditions. Along with a significant accumulation of biomass on the microcosms retrieved from the ponds, these results suggest that some change in the microbial community dynamics may operate between the anoxic soil compartments and the more aerobic ponds. Moreover, the fact that all the extracted fatty acids showed incorporation in ^{13}C suggests that the microbial community established on the microcosms were mostly involved in contaminant degradation. However, it should be considered that cross feeding by metabolites or recycling of dead biomass within the biofilm may also channel labelled carbon into individual members of the microbial community, explaining the variation in the labelling of specific fatty acids. In general, fatty acids displaying a higher incorporation of ^{13}C were very likely synthesised by organisms feeding on [$^{13}C_6$] MCB, whereas organisms synthesising non-labelled fatty acids were likely not involved in the degradation of the labelled MCB and use different carbon sources. The analysis of the composition of total fatty acid fractions showed that this method might not be sensitive enough for investigating in detail changes in microbial communities between soil and water compartments as well as down gradient the flow path.

Additionally, the MCB degradation processes in the wetland were investigated by carbon stable isotope composition analysis. A correlation between decreasing MCB concentration and a shift in the carbon isotope signature towards the heavier isotope was visible along the flow path,

suggesting the degradation of MCB. Toxic effects of MCB on the MCB-degrading population can be reasonably excluded at the observed range of concentration values (Fritz et al., 1992; Vogt et al., 2002; Vogt et al., 2004).

In the transition from the soil compartments to the ponds, a substantial contaminant mass depletion without concomitant isotope enrichment was observed. In this open system, it is likely that part of the contaminant may partition into the atmosphere, affecting the MCB concentration values without generating a significant isotope shift. Moreover, under oxic-prevailing conditions, several bacteria have the ability to use MCB as sole carbon and energy source, and may putatively adopt the well-known and described aerobic degradation pathways (Van Agteren et al., 1998). These bacteria may degrade MCB at faster rate than the degrading bacteria associated with anoxic conditions, contributing to the observed contaminant mass decrease. These oxygen-driven degradation reactions would lead to MCB mass decrease without a significant associated isotope effect (Kaschl et al., 2005). In parallel, biogeochemical processes such as oxidation of ferrous iron or mineral surfaces may compete for oxygen (Ehrlich, 1998; Søgaard et al., 2001; Warren and Haack, 2001), leading to transient conditions and oxygen gradients, which may affect the composition of the existing microbial community and rate of degradation reactions. However, the anaerobic degradation pathway of MCB is not elucidated yet.

In the anoxic soil compartments, two major hypothetical degradation pathways would come into consideration: 1) reductive dechlorination of MCB, and 2) degradation of MCB as an electron donor molecule. First, an initial dechlorination of MCB, followed by the degradation of benzene, is likely to occur in the soil compartments. Indeed, the presence of ^{13}C-labelled benzene detected in all the microcosms along with the detection of low benzene concentrations suggested that MCB is degraded reductively to benzene under anoxic conditions prior mineralisation. This pathway would suggest a similar pattern as observed previously by Nowak et al. (1996). The pH values were, however, constant, and the relatively high background level of chloride in the groundwater hindered the direct verification of MCB degradation by an increasing chloride concentration along the flow path. Bacterial breakdown of benzene could lead to the formation of benzoate or phenol as intermediates (Chakraborty and Coates, 2005; Edwards and Grbic-Galic, 1992; Lovley, 2000; Phelps et al., 2001; Ulrich et al., 2005), which were not detected in this study. Soluble organic carbon species, which could serve as electron donors for dechlorination processes, were found in low concentration in the unplanted segment, which might effectively limit the extent of degradation. Conversely, dechlorination activity in the planted segment may be partly related to the abundance of hydrogen and reduced organic acids such as acetate and propionate (Holliger et al., 1992; Middeldorp et al., 1997).

Reductive dechlorination reaction of MCB to benzene is expected to be associated with a pronounced primary isotope effect (Griebler et al., 2004a). In contrast, the isotope composition presented in this study displayed a slight but significant enrichment ranging from 0.4 to 0.7 δ units, in the soil compartments of the planted and the unplanted segments, respectively. However, the isotope effect at zones of preferential *in situ* degradation reaction can be substantially higher. If other non fractionating processes such as sorption and volatilisation also contribute to a decrease in MCB concentrations, the isotope effect upon *in situ* degradation is expected to be relatively high. The observed isotope effect points to dominating anaerobic processes, as inferred by the estimation of biodegradation levels over the flow path. In the planted, the oxygen supplied by the plant at the rizosphere level may favour the establishment of aerobic zones. Although this process is not relevant for the electron budget, oxygen may contribute to the MCB degradation reactions, leading to MCB decrease without concomitant isotope effect. A fractionating anaerobic process and a less fractionating aerobic process may both contribute to *in situ* degradation, resulting in a mixed overall fractionation at the planted segment. Conversely, the isotopic composition shift observed at the unplanted segment suggests the occurrence of a more fractionating process.

Alternatively, MCB may be degraded as an electron donor molecule under ferric iron- or sulphate-reducing conditions. Anaerobic oxidation of benzene under these conditions has been previously observed (Anderson and Lovley, 2000; Anderson et al., 1998). For instance, geochemical footprints of iron reduction processes were found, and the ferrous iron mobilisation was increasing as a function of the flow path. Dissolved Fe in pore waters can be a result of different processes such as Fe(III) reduction (Lovley, 1991; Lovley, 1997), pyrite oxidation (Lord and Church, 1983), or Fe complexation (Luther et al., 1996). Fe(II) may be precipitated with sulphide originating from sulphate reduction activity or form complexes. Therefore the concentrations may not reflect the true extent of iron reduction in the presence of sulphate reduction. A low extent of sulphate reduction process and the availability of reactive iron may prevent accumulation of H_2S in the near-neutral conditions of the wetland. A MCB mineralisation by sulphate reduction contributing to the contaminant mass decrease is feasible and may be possible. However, due to the high concentrations of sulphate in the supplied water, a reliable estimation of the extent of sulphate reduction could not be carried out. However, a reduction of ferric iron directly linked stoichiometrically to MCB oxidation in the wetland may theoretically account for a MCB mass decrease of about 18% between the inflow and the outflow of the system at both the planted and unplanted segment.

Additionally, other unknown degradation pathways can not be excluded. These hypotheses will require further detailed investigations, along with further isolation and identification of microorganisms involved in the anaerobic MCB degradation in wetlands treating MCB contaminated water.

7.7 Conclusion

The integrated approach provided evidence for *in situ* MCB biodegradation in both, soil compartments and ponds of the planted and unplanted segments of a horizontal subsurface flow constructed wetland. This was supported by isotopic fractionation analysis, combined with *in situ* microcosm experiments, which can be utilized to document further the *in situ* degradation of MCB and other contaminants in wetland systems. Further investigations to elucidate the microbial degradation of MCB, facilitated by an integrated approach and combined with a high resolution sampling, are required to evaluate zones of enhanced *in situ* biodegradation of MCB and to optimise wetland systems treating contaminated groundwater.

7.8 Acknowledgements

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8 Zusammenfassung

Die Untersuchungen, die im Rahmen dieser Promotion durchgeführt wurden, stehen im Kontext von *Natural Attenuation* (NA), den natürlichen Abbau- und Rückhalteeigenschaften eines Grundwasserleiters, die immer häufiger zur Überwachung und Sicherung kontaminierter Standorte genutzt werden. Da insbesondere der mikrobielle Schadstoffabbau zu einer destruktiven und vor allem nachhaltigen Reduzierung der Schadstoffmengen im Aquifer beiträgt, ist der Nachweis des biologischen Abbaus von Grundwasserkontaminanten ein wesentliches Element für die Implementierung von NA als Standortsicherungskonzept. Es gibt nur wenige Methoden, die einen direkten Nachweis der Biodegradation unter *in situ* Bedingungen ermöglichen. Inhalt der vorliegenden Dissertation ist daher die Entwicklung eines Testsystems (*in situ* Mikrokosmen, BACTRAP®) zum direkten Nachweis des mikrobiellen Schadstoffabbaus im kontaminierten Grundwasser sowie die Integration des Verfahrens in Monitoringstrategien für NA-Konzepte.

8.1 Beurteilung der *in situ* Mikrokosmen zum Nachweis der Biodegradation

Mit der Entwicklung von *in situ* Mikrokosmen (BACTRAP®) steht neben der substratspezifischen Isotopenfraktionierungsanalyse (CSIA) und *in situ* Tracerversuchen entlang einer Fließstrecke oder in Form von Push- und Pull Tests ein weiteres direktes Verfahren zur Charakterisierung des mikrobiellen *in situ* Abbaupotenzials in kontaminierten Aquiferen zur Verfügung. Mit *in situ* Mikrokosmen kann der mikrobielle Abbau von Grundwasserkontaminanten mit wenig Aufwand und ohne spezielle Vorkenntnisse zu den biogeochemischen Milieubedingungen direkt in einer Grundwassermessstelle untersucht werden. Die Methode eignet sich gleichermaßen für Anwendungen in konventionellen Grundwassermessstellen und für Multilevel-Untersuchungen, bei denen tiefenorientierte Aussagen bezüglich des *in situ* Abbaupotenzials gewonnen werden können (siehe Kapitel 2, 3, 5). Darüber hinaus ist der Einsatz von *in situ* Mikrokosmen nicht nur auf den Grundwasserleiter beschränkt. In Untersuchungen zum MCB Abbau in einer Pflanzenkläranlage wurden BACTRAPs erstmals direkt im Sedimentkörper inkubiert (Kapitel 7, Anhang H).

Die Verwendung ^{13}C-markierter Verbindungen und die Analyse der Fettsäurefraktionen mittels GC-C-IRMS ermöglichen einen sehr sensitiven Nachweis der mikrobiellen Abbauaktivität (Boschker and Middelburg, 2002; Zengler et al., 1999), so dass selbst geringe Stoffumsätze im unteren Nanogramm Bereich empfindlich detektiert werden können (siehe Kapitel 4, 5, 6). Damit sich ausreichend Mikroorganismen auf dem Testsystem ansiedeln und eine substanzielle ^{13}C-Anreicherung der Biomasse erfolgen kann, sollte die Inkubationsdauer der *in situ* Mikrokosmen im Grundwasser circa zwei bis drei Monate betragen. Es kann also

bereits innerhalb weniger Monate eine Charakterisierung des Abbaupotenzials an einem kontaminierten Standort vorgenommen werden, weshalb sich *in situ* Mikrokosmen insbesondere zur Untersuchung der sehr langsam ablaufenden anaeroben Prozesse, wie für den Nachweis der Biodegradation persistenter Kontaminanten (z.B. MCB), eignen (Kapitel 5 bis 7).

Bisher galt MCB unter anoxischen Bedingungen als nicht abbaubare Verbindung (Field and Sierra-Alvarez, 2008). Der Einsatz stabiler Isotope ermöglichte den sensitiven Nachweis der vollständigen Mineralisierung in Labormikrokosmen sowie die Transformation in die bakterielle Biomasse unter *in situ* Bedingungen. Dadurch konnte der anaerobe MCB-Abbau sowohl im Labor als auch unter *in situ* Bedingungen nachgewiesen werden (Kapitel 5 bis 7). Die Bestimmung der Reaktionsmechanismen sowie die Identifizierung der am anaeroben MCB-Abbau beteiligten Mikroorganismen sind Gegenstand zukünftiger Untersuchungen. Im Rahmen weiterer Tracerversuche mit isotopisch markiertem MCB könnten charakteristische Metabolite Informationen zum Abbauweg liefern und RNA- bzw. DNA SIP zur Analyse der am MCB Abbau beteiligten mikrobiellen Gemeinschaft genutzt werden.

Ziel der Untersuchungen mit *in situ* Mikrokosmen ist es, die Abbaubarkeit organischer Grundwasserkontaminanten unter möglichst natürlichen Bedingungen nachzuweisen. Entgegen den alternativen Labormethoden werden Versuche mit BACTRAPs daher direkt unter den vorherrschenden Aquiferbedingungen durchgeführt, in dem das Testsystem in einer Grundwassermessstelle installiert wird. Von Nachteil ist hierbei, dass eine Messstelle nur bedingt die Aquiferbedingungen repräsentieren kann, da das Pegelwasser über längere Zeit stagniert und deshalb nur eingeschränkt dem umgebenden Grundwasser entspricht. Eine optimierte Art der Probenahme könnte erfolgen, in dem das Testsystem stattdessen direkt im Aquifersediment inkubiert wird. Diese Art der Beprobung ist jedoch technisch aufwendig und für Routineuntersuchungen ungeeignet, insbesondere für tiefer liegende Grundwasserstockwerke.

In situ Mikrokosmen bestehen aus einem Aufwuchskörper, der den Mikroorganismen als Substratpool und als besiedelbare Oberfläche dient. Um die Ergebnisse der BACTRAP-Untersuchungen auf den Grundwasserleiter übertragen zu können, sollten die Aufwuchskörper den Bewuchs sowie die Abbauaktivität in keiner Weise stimulieren. Wie die Ergebnisse in Kapitel 4 gezeigt haben, konnten die dominierenden Organismen der Grundwassermikroflora mittels molekularbiologischer Methoden (SSCP) auch auf den *in situ* Mikrokosmen nachgewiesen werden (Fig. 4-6). Darüber hinaus stimmten die Ergebnisse der molekularbiologischen Untersuchungen sehr gut mit den geochemischen Milieubedingungen überein. Untersuchungen im Randbereich der BTEX-Fahne am Standort Zeitz zeigten eine Dominanz von Alpha- und Betaproteobakterien und die Sequenzierung ausgewählter Banden von Toluol beladenen BACTRAPs ergab gute Übereinstimmung mit *Azoarcus* sp.,

bekannt als Toluol- beziehungsweise *m*-Xylol Abbauer unter nitratreduzierenden Bedingungen (Tab. 4-2). Diese Ergebnisse korrelierten sehr gut mit der lokalen Geochemie am Fahnenrand, wo Nitrat als relevanter Elektronenakzeptor in Frage kommt.

Vergleichbare Untersuchungen mittels DNA-Fingerprint Methoden zur Phylogenie der auf den *in situ* Mikrokosmen siedelnden mikrobiellen Gemeinschaft an einem weiteren BTEX kontaminierten Standort ergaben eine Dominanz von bisher noch nicht kultivierten Mikroorganismen mit nahem Verwandtschaftsgrad zu Eisen- und Sulfatreduzierern, insbesondere der *Geobacter* und *Desulfurumonas* Gruppen. Diese Organismen sind dafür bekannt, am anaeroben Abbau organischer Kontaminanten im Grundwasser beteiligt zu sein. Da die dominierenden terminalen Elektronenakzeptoren im Aquifer Sulfat und Eisen(III) sind, konnte auch an diesem Standort eine gute Übereinstimmung zwischen der Geochemie und den molekularbiologischen Untersuchungsergebnissen gefunden werden. Die Ergebnisse verschiedener Feldversuche zur Charakterisierung der am Schadstoffabbau beteiligten Mikroorganismen haben gezeigt, dass *in situ* Mikrokosmen geeignet sind, um in Kombination mit DNA *Fingerprinting* Methoden empfindlich die mikrobielle Gemeinschaft in Übereinstimmung mit der regionalen Geochemie abzubilden. Auch die Beladung der Aufwuchskörper mit verschiedenen Substraten wie Benzol und Toluol führte im Vergleich zu unbeladenen Testsystemen zu keiner signifikanten Veränderung hinsichtlich der Zusammensetzung der mikrobiellen Gemeinschaft, wie die Fettsäureprofile und insbesondere die SSCP Analysen gezeigt haben (Kapitel 3, 4). Dies verdeutlicht, dass sich das Testsystem nicht selektiv auf die bakterielle Gemeinschaft auswirkt und im Wesentlichen kein präferentieller Abbau stattfindet; eine wichtige Voraussetzung für die Anwendung der *in situ* Mikrokosmen zur Erfassung des Abbaupotenzials kontaminierter Standorte.

Basierend auf dem Einsatz ^{13}C-markierter Substrate kann selbst in komplexen Systemen, mit multiplem Kontaminationsspektrum, anhand der isotopischen Markierung in Biomarkern die *in situ* Biodegradation für einen bestimmten Schadstoff nachgewiesen werden. Eine fundamentale Grundlage für die Anwendung des Testsystems ist jedoch, dass die Schadstoffe, deren Abbau untersucht werden soll, von den Mikroorganismen als Kohlenstoffquelle benutzt werden, da ansonsten die Biodegradation nicht mit einer Inkorporation ^{13}C-markierten Kohlenstoffs in die Biomasse verbunden ist. Daher ist der Nachweis der reduktiven Dehalogenierung (RDH) von chlorierten, bromierten oder fluorierten Verbindungen, die während der RDH als Elektronenakzeptoren genutzt werden, mit dieser Methode nicht möglich.

Die mikrobielle Abbauaktivität wird anhand der Inkorporation ^{13}C-markierten Kohlenstoffs in Biomarkermoleküle nachgewiesen. Hat eine ^{13}C-Anreicherung in den Zellbestandteilen stattgefunden, so gilt dies als eindeutiger Indikator für *in situ* Abbau. Ist keine ^{13}C-Anreicherung in der Biomasse nachweisbar, kann ein *in situ* Abbaupotenzial dennoch nicht

ausgeschlossen werden. Die Ergebnisse zeigen in diesem Fall nur, dass mithilfe der *in situ* Mikrokosmen ein Nachweis des Abbaus nicht erfolgen konnte. Verschiedene Ursachen hierfür sind denkbar. Da insbesondere unter anoxischen Bedingungen das Wachstum von Schadstoff abbauenden Mikroorganismen sehr langsam erfolgt, war möglicherweise die Inkubationszeit zu kurz, so dass sich die mikrobielle Gemeinschaft des Aquifers nicht auf dem Testsystem etablieren konnte und daher nicht ausreichend Schadstoff abbauende Mikroorganismen zur Verfügung standen. Weiterhin ist denkbar, dass Mikroorganismen die ^{13}C-markierten Substrate primär für den Energiestoffwechsel und nicht zur Synthese von Biomasse (Zellwachstum) verwenden (Thauer et al., 1977), so dass keine Inkorporation des markierten Kohlenstoffs in die Biomarker erfolgen kann. Findet eine Umsetzung der ^{13}C-markierten Substrate ausschließlich durch den Energiestoffwechsel ohne Zellwachstum statt, so könnten dennoch isotopisch markierte Metabolite als Indikator für Biodegradation auf dem Testsystem nachgewiesen werden.

Eine Quantifizierung des mikrobiellen Abbaus mithilfe von *in situ* Mikrokosmen ist schwierig, aber bedingt möglich (Kapitel 4). Anhand der Menge des ^{13}C-markierten Kohlenstoffs in den Fettsäuren kann die Menge des dafür benötigten Substrats bestimmt werden. Unter weiterer Berücksichtigung des Anteils der Fettsäuren an der Gesamtbiomasse und unter der Annahme repräsentativer Ertragskoeffizienten für den anaeroben Abbau kann schließlich die Gesamtmenge des ^{13}C-markierten Substrats ermittelt werden, die während der Inkubation des Testsystems mikrobiell umgesetzt wurde. Auf diese Art und Weise berechnete Umsatzraten für Versuche mit Benzol und Toluol ergaben, dass maximal 2,5 % des Substratverlustes auf mikrobiellen Abbau zurückgeführt werden konnten (Kapitel 4), so dass Verluste infolge von Desorption um Größenordnungen höher waren (Geyer et al., 2005). Alternativ sind semiquantitative Aussagen anhand der ^{13}C-Anreicherungen in den Fettsäuren möglich, da die Intensität der Markierung als direktes Maß der mikrobiellen Aktivität betrachtet werden kann. Abgesehen von geringen Isotopenfraktionierungsprozessen bei der Fettsäuresynthese spiegeln die Isotopensignaturen der Fettsäuren im Wesentlichen das Isotopenverhältnis des Wachstumssubstrats (Kohlenstoffquelle) wider (Abraham et al., 1998; Deniro and Epstein, 1978). Pelz et al. (2001a) zeigten in Markierungsexperimenten gleiche Isotopensignaturen von Fettsäuren verschiedener Kettenlängen und auch ein wesentlicher Einfluss der Wachstumsphasen auf die Isotopensignaturen konnte nicht beobachtet werden (Abraham et al., 1998). Der Kohlenstoff wird also gleichermaßen zum Aufbau von Membranlipiden verwendet, so dass unterschiedlich markierte Fettsäuren auf eine diverse Besiedlung durch mikrobielle Konsortien hindeuten können. Da das Testsystem im Grundwasser nicht ausschließlich von Schadstoff abbauenden Mikroorganismen besiedelt wird, muss zusätzlich mit einem Verdünnungseffekt des Isotopensignals gerechnet werden, da Fettsäuren von Bakterien, die nicht am Abbau beteiligt sind, folglich auch keine ^{13}C-

Anreicherung aufweisen. Als alternative Kohlenstoffquellen stehen den Mikroorganismen zum Beispiel die unmarkierten Aquiferkontaminanten oder gelöster organischer Kohlenstoff (DOC) zur Verfügung. Trotzdem kann anhand der Intensität der Markierung auf die Abbauaktivität geschlossen werden. Eine intensivere Inkorporation ^{13}C-markierten Kohlenstoffs deutet auf eine höhere Abbauaktivität hin. Auf dieser Ebene können an einem Standort Ergebnisse verschiedener Messstellen hinsichtlich ihrer Abbauaktivitäten miteinander verglichen werden. Beim Vergleich von Benzol- und Toluol BACTRAPs dokumentierte die deutlich höhere Anreicherung ^{13}C-markierten Kohlenstoffs in den Fettsäuren der Mikroorganismen, die die Toluol BACTRAPs besiedelten, einen bevorzugten und intensiveren Toluolabbau (Kapitel 3, (Geyer et al., 2005)). Diese Resultate stimmen gut mit früheren Studien überein, die bereits gezeigt haben, dass Toluol deutlich leichter abbaubar ist als Benzol (Cunningham et al., 2001; Da Silva and Alvarez, 2004; Wiedemeier et al., 1999). Ebenso wurden signifikante Unterschiede hinsichtlich des Abbaupotenzials zwischen hoch- und niedrig kontaminierten Bereichen der Schadstofffahne ermittelt, wobei für die weniger stark belasteten Zonen eine höhere ^{13}C-Anreicherung bestimmt wurde (Kapitel 3). Möglicherweise wirken sich die hohen Schadstoffkonzentrationen im Kontaminationszentrum hemmend auf den bakteriellen Metabolismus aus. Darüber hinaus ist zu berücksichtigen, dass das Angebot unmarkierten Substrats im hoch kontaminierten Bereich wesentlich höher ist, als am Fahnenrand oder im Anstrom. Unmarkierter Schadstoff der den Mikroorganismen als Wachstumssubstrat zur Verfügung steht, kann zu einem Verdünnungseffekt der Markierung in den Fettsäuren führen. Prinzipiell zeigen die Ergebnisse jedoch, dass das Testsystem signifikante Unterschiede in Bezug auf die Intensität des Abbaus widerspiegeln kann, so dass *in situ* Mikrokosmen zur Charakterisierung des Abbaupotenzials geeignet sind.

Die Gesamtbiomasse eines *in situ* Mikrokosmos kann sowohl aus lebender als auch toter Biomasse sowie aus abbauaktiven und nicht aktiven Mikroorganismen bestehen. Da der Nachweis der *in situ* Aktivität anhand der ^{13}C-Inkorporation in die Biomasse erfolgt, werden statt den PLFA bevorzugt die Gesamtlipide (TLFA) extrahiert. PLFA dienen als Biomarker für die lebende Biomasse (Tab. 1-2, (Green and Scow, 2000; Kaur et al., 2005)). TLFA hingegen berücksichtigen nicht nur Fettsäuren der Zellmembranen sondern auch der Speicherstoffe und eine Differenzierung zwischen lebender und toter Biomasse erfolgt nicht, so dass theoretisch die Fettsäuren aller Organismen, die am Schadstoffabbau beteiligt waren, extrahiert werden können. Da die Fettsäureprofile aus den BACTRAP-Untersuchungen (Kapitel 3 bis 7) ein geringes taxonomisches Potenzial aufwiesen, werden zur Charakterisierung der bakteriellen Gemeinschaft eher Nukleinsäure-basierende Fingerprint Verfahren empfohlen (Kapitel 3, 4).

Neben dem Nachweis der Biodegradation anhand isotopisch markierter Biomasse als direkter Indikator für mikrobielle Assimilation der Schadstoffe unter *in situ* Bedingungen, kann zusätzlich die Analyse charakteristischer Metabolite zum Nachweis des Schadstoffabbaus genutzt werden. Darüber hinaus bietet die Analyse von Metaboliten die Möglichkeit Abbauwege zu rekonstruieren. Im Rahmen eines Feldversuchs mit ^{13}C-Toluol beladenen BACTRAPs unter anoxischen Aquiferbedingungen konnte ^{13}C-markiertes Benzylsuccinat vom Aufwuchsmaterial der *in situ* Mikrokosmen extrahiert werden. Benzylsuccinat ist ein typischer Metabolit des anaeroben Toluolabbaus *via* Fumarataddition, katalysiert durch die Benzylsuccinatsynthase (Evans et al., 1992; Heider et al., 1999; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Der Nachweis von Benzylsuccinat wurde bereits zuvor als Indikator für den anaeroben Toluolabbau genutzt (Beller, 2000; Beller, 2002; Elshahed et al., 2001; Griebler et al., 2004b; Reusser and Field, 2002). Des Weiteren wurde im Rahmen eines BACTRAP Experimentes zur Bestimmung der *in situ* Biodegradation von MCB ^{13}C-markiertes Benzol (Fig. 7-3) auf den *in situ* Mikrokosmen nachgewiesen, ein Hinweis auf reduktive Dehalogenierung des MCB (Kapitel 7). Somit können zusätzlich Informationen zu den biogeochemischen Milieubedingungen während des mikrobiellen Abbaus sowie den Reaktionsmechanismen gewonnen werden, was eine bessere Charakterisierung der *in situ* Prozesse und die Rekonstruktion der Abbauwege ermöglicht. In diesem Zusammenhang ist die Reinheit der verwendeten ^{13}C-markierten Substanzen ein wichtiger Aspekt, der beachtet werden muss, damit Rückschlüsse auf potenzielle Metabolite möglich sind. Bereits geringe Unreinheiten in den ^{13}C-markierten Substraten könnten zu Fehlinterpretationen führen. So konnte beispielsweise festgestellt werden, dass ^{13}C-markiertes MCB auch Spuren von ^{13}C-markiertem Benzol enthält, was die Identifizierung von Benzol als potenziellen Metaboliten des MCB Abbaus *via* RDH erschweren kann (Kapitel 5, 7). Insofern auf den Mikrokosmen die nachweisbaren Anteile ^{13}C-markierter Metabolite im Vergleich zu den Unreinheiten der Ausgangssubstanzen höher sind, können Aussagen zu den Reaktionsmechanismen getroffen werden.

Im Rahmen dieser Promotion konnte gezeigt werden, dass *in situ* Mikrokosmen für vielfältige Anwendungen geeignet sind. Es handelt sich um ein Testsystem, dass den Nachweis der Aktivität, Struktur und Funktion von am Abbau beteiligten Mikroorganismen ermöglicht. Darüber hinaus können *in situ* Mikrokosmen als Präinokulum für Anreicherungskulturen im Labor zur Verfügung stehen, um die Aktivität aus dem Feld ins Labor zu übertragen, mit dem Ziel langfristig die Schadstoff abbauenden Organismen zu kultivieren (Kapitel 5, 6).

8.2 Vergleich mit anderen Methoden

Kapitel 1.4 gibt einen Überblick über die Methoden, die zum Nachweis der *in situ* Biodegradation zur Verfügung stehen: Überwachung der räumlichen und zeitlichen Abnahme

der Schadstoffkonzentrationen; Ausbildung charakteristischer Redoxzonen; Anreicherung spezifischer Metabolite; Labormikrokosmenstudien; molekularbiologische Techniken wie Nukleinsäure basierende Fingerprint Verfahren; substratspezifische Isotopenanalyse (CSIA) sowie *in situ* Tracerversuche mit radioaktiven oder stabilen Isotopen markierten Substraten. Mit Ausnahme der zwei zuletzt genannten Verfahren ist mit dem Großteil dieser Methoden nur ein indirekter Nachweis des *in situ* Abbaupotenzials möglich, was zur Entwicklung der *in situ* Mikrokosmen motiviert hat. Deshalb soll dieses Kapitel im Wesentlichen dazu dienen, anhand der Ergebnisse dieser Promotion eine Evaluierung und Gegenüberstellung der *in situ* Mikrokosmen mit den anderen genannten Methoden vorzunehmen.

Es ist schwierig in einer Schadstofffahne mit komplexem Kontaminantenspektrum abnehmende Schadstoffkonzentrationen mit dem Verbrauch von Elektronenakzeptoren und der damit in Verbindung stehenden Ausbildung typischer Redoxzonen zu korrelieren, um den Abbau einzelner Schadstoffe nachzuweisen. Häufig stellt die Zehrung von Elektronenakzeptoren in kontaminierten Aquiferen ein komplexes Zusammenspiel unterschiedlicher Abbauprozesse dar. Ein gleichzeitiges Auftreten von signifikanten Intermediaten könnte die Charakterisierung der biogeochemischen Prozesse erleichtern, insofern ein ursprünglicher Eintrag der Metabolite am Untersuchungsstandort ausgeschlossen werden kann. Alternativ werden häufig mikrobiologische Untersuchungen an Standortmaterial (Grundwasser, Sediment) im Rahmen von Labormikrokosmenstudien durchgeführt. Ein wesentlicher Vorteil der Laborstudien ist, dass in geschlossenen Laborsystemen Stoffbilanzen einfacher erfasst werden können. Der wesentliche Nachteil liegt in der eingeschränkten Übertragbarkeit der Ergebnisse aus dem Labor auf die weitaus komplexeren *in situ* Bedingungen kontaminierter Aquifere. Außerdem ist die Vielzahl der Schadstoffabbauer noch nicht kultivierbar und Laborversuche sowie Säulenexperimente unter quasi *in situ* Bedingungen sind sehr schwierig sowie arbeits- und zeitaufwändig, womit die Limitationen dieser Methoden deutlich werden.

Die Most-Probable-Number (MPN) und DNA-Fingerprint Methoden sind zwar für die Bestimmung von Gesamtzellzahlen bzw. zur Charakterisierung der mikrobiellen Gemeinschaften geeignet, aber allein der Nachweis über die Anwesenheit bestimmter Mikroorganismen erlaubt noch keine Rückschlüsse auf deren Abbauaktivität, so dass diese Methoden nur Aussagen zum Abbaupotenzial liefern können, ein direkter Nachweis des Abbaus jedoch nicht erfolgen kann. Ein direkter Nachweis der vollständigen Mineralisierung sowie der Transformation von Schadstoffen in die Biomasse ist allein mithilfe von stabilen bzw. radioaktiven Isotopen als Tracer möglich. Aufgrund des erhöhten Risikos, ausgehend von radioaktiven Stoffen, für den Mensch und die Umwelt werden bevorzugt Verbindungen mit stabilen Isotopen in offenen Systemen genutzt.

Eine etablierte Methode zur Charakterisierung der Abbauprozesse im Aquifer stellen *in situ* Tracerversuche dar, die entlang einer Grundwasserfließstrecke oder als *Pusch- und Pull* Tests in einer Grundwassermessstelle durchgeführt werden (Kapitel 1.4.2). Diese Methoden eignen sich, um den Schadstoffabbau anhand isotopisch markierter Biomasse, Metabolite oder Mineralisierungsprodukte nachzuweisen. Die Aussagen, die mit *in situ* Mikrokosmen oder *in situ* Tracerversuchen gewonnen werden können, sind vergleichbar. Während Tracerversuche entlang einer Fließstrecke mit umfangreichen Probenahmen zur Bestimmung der Tracer Durchbruchskurven verbunden sind, erfordern *in situ* Mikrokosmen neben dem Ein- und Ausbau keine weiteren Feldarbeiten, so dass der Arbeitsaufwand bei der Durchführung von Tracerversuchen deutlich größer ist. Darüber hinaus werden für *in situ* Tracerversuche größere Mengen isotopisch markierter Substanzen benötigt, als für die Analyse mit *in situ* Mikrokosmen. Das führt zu deutlich höheren Kosten. Ein Vorteil der Tracerversuche liegt darin, dass der Aquifer den tatsächlichen Reaktionsraum darstellt, während *in situ* Mikrokosmen innerhalb von Grundwassermessstellen inkubiert werden. Weiterhin erlauben die Tracerversuche auch eine quantitative Abschätzung der Biodegradation im Aquifer. Beide Methoden basieren auf dem Einsatz isotopisch markierter Substrate, was einen direkten Nachweis des mikrobiellen Abbaus bestimmter Kontaminanten anhand des Verbleibs des ^{13}C-markierten Kohlenstoffs ermöglicht.

Eine alternative Möglichkeit zum Nachweis der *in situ* Biodegradation bietet die Analyse der substratspezifischen Isotopenfraktionierung (CSIA) (Kapitel 1.4.1). Tracerversuche (Fischer et al., 2006; Reusser and Field, 2002) und CSIA (Anhang I) sind die derzeit einzigen Verfahren, die neben *in situ* Mikrokosmen die direkte Erfassung des mikrobiellen Schadstoffabbaus im kontaminierten Aquifer erlauben.

Unter der Voraussetzung, dass der mikrobielle Abbau zu einer signifikanten Veränderung der Isotopensignaturen in der residualen Schadstofffraktion führt, kann die *in situ* Biodegradation mittels CSIA über einen Fließweg auch quantitativ erfasst werden. Dazu ist in der Regel ein signifikanter Abbau mit einem deutlichen Konzentrationsgradienten notwendig, der vorwiegend in den Randbereichen von Schadstofffahnen beobachtet wird. Sind die hydrogeochemischen Milieubedingungen des Aquifers bekannt, kann der biologische Abbau unter Annahme charakteristischer Isotopenfraktionierungsfaktoren auch quantifiziert werden. Zur Analyse des Abbaupotenzials im hoch kontaminierten Bereich der Schadstoffquelle reicht die Empfindlichkeit dieser Methode oft nicht aus, da das Abbausignal sehr stark durch die hohen Anteile von nicht abgebauten Schadstoffen überlagert wird, wie im Rahmen eines Multilevel-Experimentes zur Untersuchung der vertikalen Struktur einer Benzolfahne gezeigt werden konnte (Kapitel 2,3). In den zentralen Bereichen der Fahne mit maximalen Schadstoffkonzentrationen lieferten die Benzolisotopensignaturen keinen Hinweis auf Benzolabbau, während mithilfe von *in situ* Mikrokosmen anhand der Inkorporation ^{13}C-

markierten Kohlenstoffs auch im hoch kontaminierten Bereich der Schadstofffahne Biodegradation nachweisbar war.

Für Schadstoffe mit großen Molekülen (> 12 Kohlenstoffatome), z.B. höher molekulare PAKs, ist CSIA nicht geeignet, da der messbare Isotopeneffekt zu gering ist, um sensitiv genug nachgewiesen werden zu können (Meckenstock et al., 2004a). Eine weitere Schwierigkeit stellen heterogene Schadstoffquellen und überlagerte Schadstofffahnen dar, weil es auch zu einer Überlagerung der Isotopensignale kommen kann. Abbaureaktionen, die prinzipiell nicht mit einer signifikanten Isotopenfraktionierung verbunden sind, wie teilweise für den aeroben MCB- und Toluolabbau beobachtet wurde (Kaschl et al., 2005; Morasch et al., 2002; Sherwood Lollar et al., 1999), können folglich nicht mittels CSIA erfasst werden. In diesem Fall können *in situ* Mikrokosmen einen wesentlichen Beitrag zur Charakterisierung der *in situ* Biodegradation leisten. Wie weiterhin in Kapitel 6 für chlorierte Benzole gezeigt wurde, stellen sequenzielle Abbauwege wie die RDH ein zusätzliches Problem für die Anwendung des CSIA Konzeptes dar, weil Intermediate sowohl durch Bildung als auch mikrobiellen Abbau, verbunden mit einem gegensätzlichen Isotopeneffekt, beeinflusst werden. In diesem Fall können durch die Berechnung einer kumulativen Isotopenbilanz Informationen zum Abbaupotenzial erhalten werden. Dennoch sind ergänzende Untersuchungen zur Verifizierung der Ergebnisse hilfreich, insbesondere da für die Beurteilung des *in situ* Abbaupotenzials kontaminierter Standorte mehrere Beweislinien gefordert werden.

Wie in den verschiedenen Feldstudien zur Evaluierung und Verifizierung des Testsystems gezeigt werden konnte, liefern *in situ* Mikrokosmen Ergebnisse, deren Plausibilität anhand der Übereinstimmung mit Ergebnissen anderer Methoden überprüft wurde. Die Analyse der vertikalen Struktur einer Benzolfahne mit geochemischen und isotopenchemischen Methoden ergab ein deutliches Abbaupotenzial für Benzol, was durch den Einsatz von *in situ* Mikrokosmen verifiziert werden konnte (Kapitel 2,3). Ist der Einsatz alternativer Methoden wie CSIA oder *in situ* Tracerversuchen unter bestimmten Umständen limitiert, so können *in situ* Mikrokosmen weiterführende Informationen zur Charakterisierung der *in situ* Biodegradation liefern, insbesondere für den hoch kontaminierten Bereich von Schadstofffahnen oder an komplexen Standorten. Abgesehen von Tracerversuchen existieren keine Methoden, mit denen der biologische Schadstoffabbau an kontaminierten Standorten mit vergleichbarer Sensitivität nachgewiesen werden kann.

8.3 Implementierung in die Altlastenpraxis

Verschiedene Prototypen des Testsystems wurden bisher an unterschiedlichen Untersuchungsstandorten im Rahmen von Industriekooperationen erfolgreich erprobt und es

liegen Erfahrungen zum Einsatz der BACTRAPs für verschiedene Kontaminanten wie Benzol, Toluol, MCB, MTBE und PAK vor (Tab. 8-1).

Die Zusammenarbeit mit der Industrie und Umweltbehörde ermöglichte es, dieses Forschungsvorhaben bereits von Beginn an in einen angewandten Kontext zu stellen und so die Belange von Wissenschaft und Praxis miteinander zu verknüpfen. Untersuchungen mit *in situ* Mikrokosmen, die in Kooperation mit einem Industriepartner an einem ehemaligen Kokereistandort durchgeführt wurden, haben dazu beigetragen, den Standort in ein *Natural Attenuation* Vorhaben zu überführen. In diesem Vorhaben wird auf aktive Sanierungsmaßnahmen über einen bestimmten Zeitraum verzichtet, um die Stabilität der Schadstofffahne und die *in situ* Abbauprozesse zu untersuchen sowie ein Grundwassermonitoring- und Überwachungskonzept zu entwickeln. *Natural Attenuation* wurde für einen Zeitraum von 5 Jahren genehmigt, mit der Auflage weitere Monitoring-Untersuchungen durchzuführen, um den Standort zu erkunden. Im Rahmen dieser umfangreichen Standorterkundungen wurden BACTRAP Untersuchungen zum geforderten Nachweis der *in situ* Biodegradation eingesetzt. Auch die Untersuchungen zum MCB Abbau (Kapitel 5) erfolgten im Einverständnis mit der verantwortlichen Umweltbehörde. Die Methode der *in situ* Mikrokosmen ist bereits Bestandteil verschiedener Leitfäden und Handlungsempfehlungen (KORA, 2007; KORA, 2008; LABO, 2005), was die Bedeutung dieses Nachweisverfahren im Bereich der Altlastenbewertung verdeutlicht. Anhand dieser Beispiele kann gezeigt werden, dass es seitens der Behörden bereits eine Akzeptanz für diese neuartige Methode zum geforderten Nachweis der *in situ* Biodegradation an kontaminierten Standorten gibt.

Entsprechend der verschiedenen Handlungsempfehlungen und Arbeitshilfen zur Anwendung von *Monitored Natural Attenuation* (MNA) als Standortsicherungsmaßnahme kontaminierter Aquifere werden mehrere Beweislinien über den Nachweis der *in situ* Biodegradation gefordert (Hessisches Landesamt für Umwelt und Geologie, 2004; ITVA, 2004; KORA, 2007; US-EPA, 1999). *In situ* Mikrokosmen können in diesem Zusammenhang einen wertvollen Beitrag zur Charakterisierung der natürlichen Abbauprozesse liefern, um *Monitored Natural Attenuation* als Standortsicherungsmaßnahme zu implementieren. Im Gegensatz zu einer Vielzahl herkömmlicher Verfahren (siehe Kapitel 1.4) handelt es sich bei dem Testsystem um ein innovatives Nachweisverfahren, welches innerhalb weniger Monate einen direkten Nachweis der *in situ* Biodegradation ermöglicht. Die Methode kann problemlos in das Monitoring-Programm implementiert werden, ohne Erfordernis zusätzlicher Maßnahmen.

Tab. 8-1: Anwendungsbeispiele von *in situ* Mikrokosmen (BACTRAP®)

Kontaminante	Standort	Forschungsinstitut/ Industriepartner	Referenz
Benzol	Zeitz	UFZ	(Büning et al., 2005); (Geyer et al., 2005); (Kästner et al., 2006); (Stelzer et al., 2006a); (Stelzer et al., 2006b) Kapitel 2, 3, 4
Benzol	Gneisenau	DMT	(Büning et al., 2005); (Stelzer et al., 2006a) Dokumentation in internen Gutachten, (2007)
Benzol	Hansemann	DMT	Dokumentation in internen Gutachten, (2005, 2008)
Benzol	C.Rauxel	DMT	Dokumentation in internem Gutachten, (2008)
Benzol	Duisburg	HPC	in Bearbeitung, (2008)
Benzol	Gölzau	UFZ	in Bearbeitung, (2008)
Toluol	Zeitz	UFZ	(Geyer et al., 2005); (Kästner et al., 2006); (Stelzer et al., 2006a) Kapitel 3, 4
Toluol	Gneisenau	DMT	(Büning et al., 2005); (Stelzer et al., 2006a)
Toluol	C. Rauxel	DMT	Dokumentation in internem Gutachten, (2008)
Naphthalin	Hansemann	DMT	Dokumentation in internem Gutachten, (2007)
Naphthalin	Gneisenau	DMT	Dokumentation in internem Gutachten, (2007)
Naphthalin	C. Rauxel	DMT	Dokumentation in internem Gutachten, (2008)
Naphthalin	Hanau	HPC	Dokumentation in internem Gutachten, (2005)
Naphthalin	Karlsruhe	Roth und Partner	Dokumentation in internem Gutachten, (2007)
Naphthalin	Duisburg	HPC	in Bearbeitung, (2008)
Acenaphthen	Gneisenau	DMT	Dokumentation in internem Gutachten, (2007)
Acenaphthen	Karlsruhe	Roth und Partner	Dokumentation in internem Gutachten, (2007)
Anthracen	Hanau	HPC	Dokumentation in internem Gutachten, (2005)
Phenanthren	Hanau	HPC	Dokumentation in internem Gutachten, (2005)
MTBE	Leuna	UFZ	Dokumentation in internem Gutachten, (2004)
MTBE	vertraulich	GICON	Dokumentation in internem Gutachten, (2005)
MCB	Bitterfeld	UFZ	(Nijenhuis et al., 2007) Kapitel 5 (Braeckevelt et al., 2007a; Braeckevelt et al., 2007b) Kapitel 7, Anhang H
MCB	Hamburg	Roth und Partner	(Stelzer et al., 2008) Kapitel 6
Hexadekan	Gölzau	UFZ	in Bearbeitung, (2008)

8.4 Ausblick

Im Rahmen dieser Promotion wurde gezeigt, dass mithilfe von *in situ* Mikrokosmen die Aktivität, Struktur und Funktion von Schadstoff abbauenden Mikroorganismen analysiert werden kann. Damit steht ein weiteres innovatives Verfahren zum Nachweis der *in situ* Biodegradation kontaminierter Grundwasserleiter in der Altlastenpraxis zur Verfügung. Der Großteil der bisherigen Untersuchungen und Feldanwendungen wurde für Benzol, Toluol und MCB durchgeführt (Kapitel 2 bis 7, Tab. 8-1). Zukünftig soll das Testsystem für Anwendungen an PAK oder MTBE kontaminierten Standorten optimiert werden. Diese Verbindungen zählen zu den häufigsten Grundwasserkontaminanten und insbesondere für höher molekulare PAKs gibt es derzeit keine alternativen Methoden zum direkten Nachweis der *in situ* Biodegradation. Die verschiedenen Schadstoffe unterscheiden sich deutlich hinsichtlich ihrer Eigenschaften (z.B. Sorption, Wasserlöslichkeit, Bioverfügbarkeit). Schwierigkeiten zeigten sich hierbei vor allem infolge der stark sorptiven Eigenschaften der PAKs auf den aktivkohlehaltigen Trägermatierialien, was die Extraktion und Analyse der Biomasse von den *in situ* Mikrokosmen erschwert, so dass alternative Trägermaterialien benötigt werden. Erste Untersuchungen zum Einsatz der *in situ* Mikrokosmen zum Nachweis des PAK Abbaus wurden durchgeführt, wobei Teflonfilter als Aufwuchskörper dienten. Anhand der ^{13}C-Anreicherung in den Fettsäuren und Aminosäuren konnte der *in situ* Abbau von Naphthalin unter anoxischen Aquiferbedingungen nachgewiesen werden.

Ein weiterer Schwerpunkt zukünftiger Untersuchungen mit *in situ* Mikrokosmen ist die Identifikation der am Schadstoffabbau beteiligten Mikroorganismen mithilfe der Methode des DNA- oder RNA-SIP. In bisherigen Feldversuchen war die ^{13}C-Anreicherung in den Biomarkern, der auf den *in situ* Mikrokosmen siedelnden Mikroorganismen, nicht ausreichend. Für eine optimale Auftrennung von schwerer und leichter Nukleinsäurefraktion mithilfe der Dichtegradientenzentrifugation wird nach bisherigem Stand des Wissens eine deutlich höhere Inkorporation ^{13}C-markierten Kohlenstoffs von rund 30 Atomprozent benötigt (Radajewski et al., 2003).

9 Literatur

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11 Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit über die *Entwicklung eines Testsystems zum Nachweis der in situ Biodegradation im Grundwasser* von mir selbständig angefertigt wurde, abgesehen von der Beratung durch meine Betreuer und unter Verwendung der entsprechend gekennzeichneten Hilfsmittel und Literaturangaben. Mit Ausnahme der benannten Teile ist die Arbeit noch nicht veröffentlicht und wurde bisher nicht an anderer Stelle im Rahmen eines Prüfungsverfahrens eingereicht.
Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis entstanden.

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13 Publikationsliste

13.1 Artikel

1. Stelzer, N., Fischer, A., Kästner, M., Richnow, H.-H. (2006): Analyse des anaeroben *in situ* Benzolabbaus: Vergleich von In-situ-Mikrokosmen, Elektronenakzeptorbilanzen und Isotopenfraktionierungsprozessen. Grundwasser 11(4), S. 247-258.

2. Stelzer, N., Büning, C., Pfeifer, F., Tebbe, C. C., Nijenhuis, I., Kästner, M., Richnow, H.-H. (2006): *In situ* microcosms (BACTRAP) to evaluate natural attenuation potentials in contaminated aquifers. Organic Geochemistry 37(10), S. 1394-1410.

3. Fischer, A., Stelzer, N., Eisenmann, H., Richnow, H.-H. (2006): Nachweis des mikrobiellen Schadstoffabbaus in Grundwasserleitern: Innovative Isotopenverfahren erfassen Abbauprozesse direkt im kontaminierten Grundwasser. Terra Tec 15(1-2), S. 14-17.

4. Kästner, M., Fischer, A., Nijenhuis, I., Geyer, R., Stelzer, N., Bombach, P., Tebbe, C. C., Richnow, H.-H. (2006): Assessment of Microbial *In Situ* Activity in Contaminated Aquifers. Engineering in Life Science 6(3), S. 234-251.

5. Braeckevelt, M., Rokadia, H., Imfeld, G., Stelzer, N., Paschke, H. Kuschk, K., Kästner, M., Richnow, H.-H., Weber, S. (2007): Assessment of *in situ* biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland. Environmental Pollution 148(2), S. 428-437.

6. Nijenhuis, I., Stelzer, N., Kästner, M., Richnow, H.-H. (2006): Sensitive detection of anaerobic monochlorobenzene degradation using stable isotope tracers. Environmental Science and Technology 41(11), S. 3836-3842.

7. Braeckevelt, M., Rokadia, H., Mirschel, G., Weber, S., Imfeld, G., Stelzer, N., Kuschk, K., Kästner, M., Richnow, H.-H. (2007): Biodegradation of chlorobenzene in a constructed wetland treating contaminated groundwater. Water Science & Technology 56(3), S. 57-62.

8. Fischer, A., Theuerkorn, K., Stelzer, N., Gehre, M., Thullner, M., Richnow, H.-H. (2007): Applicability of Stable Isotope Fractionation Analysis for the Characterization of Benzene Biodegradation in a BTEX-contaminated Aquifer. Environmental Science and Technology 41(10), S. 3689-3696.

9. Stelzer, N., Imfeld, G., Thullner, M., Lehmann, J., Poser, A., Richnow, H.-H., Nijenhuis, I. (2008): Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers. Environmental Science and Technology, submitted.

13.2 Konferenzvorträge

1. Nijenhuis, I., Stelzer, N., Fischer, A., Kästner, M., Richnow, H.-H.: Isotope concepts to assess natural attenuation in contaminated aquifers. The General Assembly of the European Geosciences Union (EGU), 24.-29.04.2005, Vienna, Austria.

2. Stelzer, N., Geyer, R., Kästner, M., Nijenhuis, I., Weber, S., Büning, C., Pfeifer, F., Tebbe, C., Richnow, H.-H.: Erfahrungen aus dem Einsatz von ^{13}C-beladenen

Aufwuchskörpern (BACTRAP®) bei BTEX-Schadensfällen. KORA-Workshop mit Posterpräsentation “Perspektiven molekularer und isotopischer Methoden zum Nachweis des natürlichen Schadstoffabbaus in Böden, 29.-30.09.2005, FAL, Braunschweig, Deutschland.

3. Stelzer, N., Weber, S., Nijenhuis, I., Kästner, M., Richnow, H.-H.: Monitoring of *in situ* biodegradation of groundwater contaminants using a test system (BACTRAP®) with ^{13}C-labelled substrates. GASIR Jahrestagung 10.-12.10.2005 in Jena, Deutschland.

4. Stelzer, N., Fischer, A., Nijenhuis, I., Gehre, M., Kästner, M., Richnow, H.-H.: Aktuelle Entwicklungen im Bereich MNA: Nachweis des mikrobiellen Schadstoffabbaus mit Isotopenmethoden. Jahrestreffen der ITVA Regionalgruppe Bayern, 16.02.2006 in München, Deutschland.

5. Kästner, M., Fischer, A., Nijenhuis, I., Geyer, R., Stelzer, N., Bombach, P., Tebbe, C. C., Richnow, H.-H.: Assessment of microbial *in situ*-activity in contaminated aquifers. International Conference on Environmental Biotechnology (ISEB, ESEB, JSEB), 09.-13.07.2006, Leipzig, Germany.

6. Stelzer, N., Richnow, H.-H.; Nijenhuis, I: Lines of Evidence for anaerobic MCB Degradation in contaminated Groundwater based on Stable Isotope Tools. EGU European Geosciences Union, 15.-20.04.2007, Vienna, Austria.

7. Kästner, M., Fischer, A., Nijenhuis, I., Geyer, R., Stelzer, N., Bombach, P., Tebbe, C.C., Richnow, H.-H.: *In situ* microbial activity assessment in contaminated aquifers. EGU European Geosciences Union, 15.-20.04.2007, Vienna, Austria.

8. Stelzer, N., Nijenhuis, I., Lehman, J., Richnow, H.-H.: Isotopenmethoden zur Evaluierung des *Natural Attenuation* Potenzials an einem MCB kontaminierten Standort. 9. Symposium Natural Attenuation, 21.-22.11.2007 in Frankfurt, Deutschland.

9. Stelzer, N., Weber, S., Nijenhuis, I., Richnow, H.-H.: Monitoring of *in situ* biodegradation in contaminated aquifers using *in situ* microcosms (BACTRAP®) with ^{13}C-labelled substrates. AXIOM-VIBE-eTRAP-HIGRADE Workshop: "Electron transfer processes at biogeochemical gradients", 04.-07.03.2008, Leipzig, Germany.

10. Stelzer, N., Nijenhuis, I., Weber, S., Fischer, A., Richnow, H.-H.: Einsatz stabiler Isotope zum qualitativen und quantitativen Nachweis des biologischen Schadstoffabbaus im kontaminierten Grundwasser. Tagung der FH-DGG, 21.05.-25.05.2008 in Göttingen, Deutschland.

13.3 Poster

1. Stelzer, N., Geyer, R., Fischer, A., Kästner, M., Weber, S., Richnow, H.-H.: Monitoring of in-situ biodegradation using "BACTRAPs" with ^{13}C-labelled substrate. Poster presentation "1st German-American Workshop on Biogeochemical Gradients" 04.-06.05.2005 in Tübingen, Germany.

2. Stelzer, N., Kästner, M., Fischer, A., Richnow, H.-H.: Monitoring of *in-situ* biodegradation using a test system (BACTRAP) with ^{13}C-labelled substrates. Poster presentation “2nd European Conference on Natural Attenuation, Soil and Groundwater Risk Management” 18.-20.05.2005 in Frankfurt, Germany.

3. Geyer, R.; Stelzer, N., Peacock, A., White D. C., Tebbe, C. C., Richnow, H.-H., Kästner, M.: Assessment of microbial *in situ* activity using microcosm amended with

^{13}C-labelled substrates. Poster presentation "2nd European Conference on Natural Attenuation, Soil and Groundwater Risk Management" 18.-20.05.2005 in Frankfurt, Germany.

4. Richnow, H.-H., Stelzer, N., Nijenhuis, I., Kästner, M., Weber, S., Pfeifer, F., Tebbe, C. C.: In-Situ Microcosms to Assess Natural Attenuation in Contaminated Aquifers. Poster presentation International Symposia for Subsurface Microbiology (ISSM 2005) and Environmental Biogeochemistry (ISEB XVII), 14.-19.08.2005, Jackson Hole, Wyoming, USA.

5. Stelzer, N., Geyer, R., Kästner, M., Fischer, A., Weber, S., Richnow, H.-H. „Monitoring of *in situ* biodegradation in contaminated aquifers using BACTRAPs with ^{13}C-labelled substrates. Poster presentation International Summer School: "Biomonitoring, bioavailability and microbial transformation of pollutants in sediments and approaches to stimulate their biodegradation". 12-14.09.2005, Genoa, Italy.

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10. Nijhenhuis, I., Stelzer, N., Nikolausz, M., Kästner, M., Richnow, H.-H.: Evidence for microbial Degradation of Monochlorobenzene under anaerobic conditions. Poster presentation at the 11th International Symposium on Microbial Ecology (ISME), 20.-25.08.2006 in Vienna, Austria.

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12. Stelzer, N., Weber, S., Nijenhuis, I., Kuntze, K., Kästner, M., Richnow, H.-H.: Monitoring of microbial activity, metabolites and degradation pathways in contaminated aquifers using *in situ* microcosms with ^{13}C-labelled substrates. Poster presentation at the 3. European Conference on Natural Attenuation and In-Situ Remediation, 19.-21.11.2007 in Frankfurt, Germany.

14 Anhang

A **Analyse des anaeroben Benzolabbaus: Vergleich von *In situ* Mikrokosmen, Elektronenakzeptorbilanzen und Isotopenfraktionierungsprozessen (2006)**
(Grundwasser 11(4), S. 247-258)

B **Nachweis des mikrobiellen Schadstoffabbaus in Grundwasserleitern (2006)**
(Terra Tec 15(1-2), S. 14-17)

C **In situ microcosms to evaluate natural attenuation potentials in contaminated aquifers (2006)**
(Organic Geochemistry 37(10), S. 1394-1410)

D **Assessment of Microbial In Situ Activity in Contaminated Aquifers (2006)**
(Review in Engineering in Life Science 6(3), S: 234-251)

E **Sensitive Detection of Anaerobic Monochlorobenzene Degradation Using Stable Isotope Tracers (2007) und Supporting Information**
(Environmental Science and Technology 41(11), S. 3836-3842)

F **Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers (2008) und Supporting Information**
(Environmental Science and Technology submitted, 18.04.2008)

G **Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland (2007)**
(Environmental Pollution 148(2), S. 428-437)

H **Biodegradation of chlorobenzene in a constructed wetland treating contaminated groundwater (2007)**
(Water Science and Technology 56(3), S. 57-62)

I **Applicability of Stable Isotope Fractionation Analysis for the Characterization of Benzene Biodegradation in a BTEX-contaminated Aquifer (2007) und Supporting Information**
(Environmental Science and Technology 41(10), S. 3689-3696)

Anhang A

Analyse des anaeroben Benzolabbaus: Vergleich von *In situ* Mikrokosmen, Elektronenakzeptorbilanzen und Isotopenfraktionierungsprozessen (2006)

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Grundwasser 11(4), S. 247-258

Analyse des anaeroben Benzolabbaus: Vergleich von In-situ-Mikrokosmen, Elektronenakzeptorbilanzen und Isotopenfraktionierungsprozessen

Nicole Stelzer, Anko Fischer, Matthias Kästner, Hans-Hermann Richnow

Kurzfassung

Geochemische und isotopenchemische Methoden wurden zur Charakterisierung des anaeroben Benzolabbaus am Standort eines ehemaligen Hydrierwerkes (Zeitz, Deutschland) genutzt. Im Abstrom des Schadenszentrums wurde hierfür die vertikale Struktur der Benzolfahne in verschiedenen Tiefen untersucht. Durch Einsatz von [$^{13}C_6$]-Benzol in In-situ-Mikrokosmen konnte anhand der Transformation des markierten Kohlenstoffs in die Biomasse eindeutig das Abbaupotenzial nachgewiesen werden. Das Fettsäurespektrum sowie deren Isotopensignaturen deuten auf eine Besiedlung durch komplexe mikrobielle Gemeinschaften hin, die in unterschiedlicher Weise am Benzolabbau beteiligt sind. Die Sulfatsenke in der Schadstofffahne deutet auf überwiegend sulfatreduzierende Abbaubedingungen mit einem Abbaupotenzial von etwa 1,7 mmol/l Benzol hin. Anhand der Isotopensignaturen und Konzentrationen des DIC wurde in der Schadstofffahne ein Abbau von 2,2 mmol/l Benzol abgeschätzt. Eine Berechnung des Benzolabbaus anhand der Isotopenfraktionierungsmethode ergibt einen Abbau von etwa 3,0 mmol/l. Die quantitativen geochemischen und isotopenchemischen Abschätzungen liegen in der gleichen Größenordnung und zeigen einen signifikanten Benzolabbau im Aquifer.

Abstract

In situ microcosms, geochemical and isotope techniques to characterise anaerobic benzene degradation

Geochemical and isotope chemical methods were applied to assess the in situ biodegradation of benzene in a shallow aquifer (Zeitz, Germany). The vertical structure of the plume was investigated in a multi level sampling approach. Benzene degradation was investigated using in situ microcosms incubated with [$^{13}C_6$]-labelled benzene. The transformation of ^{13}C into fatty acids proved biodegradation was occurring, and indicated that a complex microbial community is colonising the in situ microcosms.

The depletion of sulphate in the plume indicated sulphate-reducing conditions may account for the oxidation of 1.7 mmol/l benzene. The concentration and isotopic composition of dissolved inorganic carbon indicated a degradation of 2.2 mmol/l benzene within the plume. The isotopic composition of benzene suggested an average degradation of 3.0 mmol/l benzene. The various approaches used to assess benzene degradation were within the same order, and illustrated significant benzene degradation in the anaerobic aquifer.

Einleitung

Benzol, Toluol, Ethylbenzol und Xylole (BTEX) sind häufige Grundwasserkontaminanten, die eine Gefahr für Ökosysteme und die menschliche Gesundheit darstellen. In einem verunreinigten Aquifer wird der Verbleib dieser Verbindungen durch abiotische (Advektion, Dispersion, Verflüchtigung, Sorption) und biologische Prozesse beeinflusst. Eine nachhaltige Reduzierung der Schadstoffmenge erfolgt allerdings hauptsächlich durch den mikrobiellen Abbau. Bei der Anwendung von Sanierungsstrategien wie Monitored Natural Attenuation (MNA) und Enhanced Natural Attenuation (ENA) kommt somit dem mikrobiellen Schadstoffabbau eine besondere Bedeutung zu. Es werden daher geeignete Methoden benötigt, um das natürliche Selbstreinigungspotenzial eines kontaminierten Grundwasserleiters bestimmen zu können.

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Eine Möglichkeit zur Untersuchung des In-situ-Abbaus von Schadstoffen ist die Nutzung der substratspezifischen Isotopenfraktionierung (Meckenstock et al. 2004). Diese Methode beruht darauf, dass Bakterien infolge des mikrobiellen Schadstoffabbaus das natürliche Isotopenverhältnis (z. B. $^{13}C/^{12}C$) eines Substrates verändern. Die bevorzugte Verwertung der leichten Isotopologe einer Kontaminante führt infolge der mikrobiellen Umsetzung zu einer Anreicherung der schweren Isotopologe in der residualen Schadstofffraktion, was mithilfe der Gaschromatographie-Isotopenverhältnis-Massenspektroskopie (GC-IRMS) analytisch nachgewiesen werden kann.

Zur Abschätzung der Biodegradation in kontaminierten Grundwasserleitern werden verschiedene Verfahren angewendet, die häufig auf der Kultivierung von Mikroorganismen im Labor beruhen. Der anaerobe Schadstoffabbau ist in der Regel ein sehr langsam ablaufender Prozess und viele der am Abbau beteiligten Mikroorganismen sind bisher noch nicht oder nur eingeschränkt kultivierbar. Labortestverfahren erscheinen daher nur begrenzt geeignet zu sein, um die realen, sehr komplexen Vorgänge und anoxischen Milieubedingungen des fließenden Grundwassers abzubilden. Darüber hinaus sind anaerobe Abbauversuche sehr langwierig und generell mit einem relativ großen Zeitaufwand verbunden.

Hieraus resultiert ein großer Bedarf an der Entwicklung geeigneter In-situ-Methoden zur Bestimmung der im Grundwasserleiter stattfindenden Biodegradation, insbesondere für anoxische Milieubedingungen.

Der mikrobielle Abbau aller BTEX-Verbindungen konnte unter oxischen und anoxischen Bedingungen nachgewiesen werden (Cerniglia 1984, Chakraborty & Coates 2004, Gibson & Subramanian 1984, Wiedemeier et al. 1999). Aufgrund der Sauerstofflimitation findet in kontaminierten Grundwasserleitern meist eine anaerobe Biodegradation der monoaromatischen Kohlenwasserstoffe statt (Lovley 2001). Dabei ist Benzol im Vergleich zu Toluol, Ethylbenzol und Xylolen oft schwerer abbaubar, sodass folglich bis heute nur wenige anaerob benzolabbauende Konsortien und eine Reinkultur isoliert wurden (Chakraborty & Coates 2004, Coates et al. 2001, Wiedemeier et al. 1999). Das begründet, warum in Laborstudien der anaerobe Benzolabbau bisher nur relativ selten eindeutig nachgewiesen werden konnte und sich in Kulturversuchen nur schwer reproduzieren lässt.

Tracerexperimente mit isotopisch markierten Substanzen stellen eine alternative Methode zur Untersuchung der Biodegradation im Grundwasser dar. Radioaktiv markierte Substanzen (^{14}C) sollten aufgrund ökologischer Risiken nicht in offenen Systemen eingesetzt werden. Ersatzweise dienen stabile Isotope (D, ^{13}C) als Tracer. Während der Assimilation wird der isotopisch markierte Kohlenstoff von den Mikroorganismen zur Synthese von Biomasse verwendet. Durch den Einsatz von ^{13}C-markierten Substraten kann der mikrobielle Schadstoffabbau anhand der Anreicherung des ^{13}C-markierten Kohlenstoffs in verschiedenen Zellkomponenten nachgewiesen werden. Dabei stehen als Zellmembranbestandteile häufig Phospholipid-Fettsäuren (phospholipid-derived fatty acids, PLFA) im Mittelpunkt der Untersuchungen (Abraham et al. 1998, Boschker & Middelburg 2002, Taylor & Parkes 1983). Es sind nur wenige Arbeiten zur Nutzung ^{13}C-markierter Substrate für die Bestimmung des mikrobiellen Schadstoffabbaus anhand der ^{13}C-Anreicherung in PLFA bekannt. Die Anreicherung von ^{13}C in verschiedenen PLFA diente zum Nachweis des Toluolabbaus. Dabei wurde Aquifermaterial in Labormikrokosmen mit [^{13}C]-α-Toluol als Substrat inkubiert, die Transformation des Isotopensignals in die Biomasse nachgewiesen, und die am anaeroben Toluolabbau beteiligten Mikroorganismen identifiziert (Pelz et al. 2001a, Pelz et al. 2001b). In ähnlichen Studien mit [$^{13}C_6$]-markiertem Toluol konnten anhand des Einbaus von ^{13}C in PLFA die mikrobiellen Populationen in aeroben Bodenmikrokosmen identifiziert werden (Hanson et al. 1999). In einem „Push & Pull"-Tracerexperiment mit [2-^{13}C]-Acetat als Substrat und Nitrat als Elektronenakzeptor ist anhand der Transformation der ^{13}C-Markierung in die verschiedenen PLFA die mikrobielle Abbauaktivität im Aquifer nachgewiesen worden (Pombo et al. 2002). Demgegenüber wurden spezielle In-situ-Mikrokosmen (BACTRAP®) entwickelt, die mit ^{13}C-markierten Testsubstanzen beladen und direkt in situ im Aquifer inkubiert werden können. Anhand der Transformation der isotopischen Markierung in die Biomasse (Membranfettsäuren) kann der Schadstoffabbau nachgewiesen werden. Zur Erprobung dieses Testsystems wurden verschiedene Experimente durchgeführt (Büning et al. 2005, Geyer et al. 2005).

Die vorliegende Arbeit stellt die Ergebnisse eines Feldversuches vor, bei dem BACTRAPs zur Analyse des anaeroben Benzolabbaus genutzt wurden. Dabei sind die Mikrokosmen mit [$^{13}C_6$]-Benzol als Substrat beladen und mithilfe eines Multilevel-Packersystems (MLPS) in acht verschiedenen Tiefen einer Grundwassermessstelle eines BTEX-kontaminierten Aquifers während 51 Tagen inkubiert worden. Der Einbau der ^{13}C-Atome aus dem Benzol in bakterielle Fettsäuren wurde im Anschluss mittels GC-IRMS nachgewiesen und als Beweis für den In-situ-Benzolabbau genutzt. Hydrogeo- und isotopenchemische Parameter dienten zur Charakterisierung des Abbaumilieus. Darüber hinaus wurde der In-situ-Benzolabbau mithilfe der Isotopenfraktionierungsmethode und anhand von Elektronenbilanzen abgeschätzt. Die Diskussion der Ergebnisse erfolgte im Hinblick auf den Einsatz verschiedener Methoden zur Kennzeichnung des Natural Attenuation Potenzials.

Material und Methoden

Chemikalien

Es wurden ausschließlich Chemikalien in analytischer Qualität (p. A.) verwendet. Isotopisch markiertes [$^{13}C_6$]-Benzol wurde von Sigma-Aldrich (St. Louis, USA) mit einer chemischen Reinheit von 99 % bezogen.

Standortbeschreibung

Bei dem Untersuchungsstandort handelt es sich um einen BTEX-Schadensfall im Bereich eines ehemaligen Hydrierwerkes bei Zeitz (Sachsen-Anhalt, Deutschland). Am Standort sind im Wesentlichen zwei Grundwasserleiter ausgebildet, die durch einen eozänen Braunkohle-führenden Ton-Schluff-Komplex voneinander getrennt sind, jedoch lokal teilweise infolge Erosion des Stauerkomplexes in direktem hydraulischem Kontakt stehen. Die Geschichte, Hydrogeologie und Geochemie des Standortes wurden ausführlich beschrieben (Dahmke et al. 2004, Fischer et al. 2004, Gödeke et al. 2004a, Gödeke et al. 2004b, Schirmer et al. 2006, Vieth et al. 2001, Vieth et al. 2005). Benzol stellt mit einem Anteil von mehr als 99 % die Hauptkontaminante dar, wobei im Zentrum der Schadstofffahne Konzentrationen von > 1.000 mg/l bestimmt wurden. Der mikrobielle BTEX-Abbau ist überwiegend

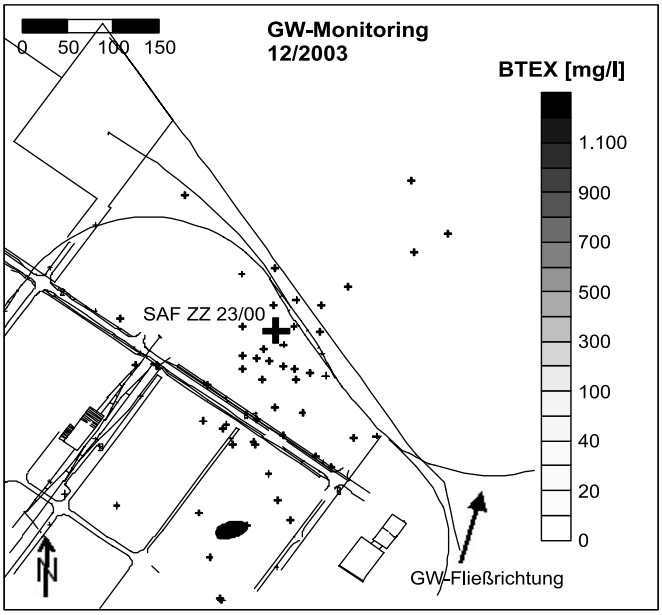

Abb. 1: Verteilung der BTEX-Konzentrationen (µg/l) im oberen Aquifer im Bereich des ehemaligen Hydrierwerkes (Zeitz, Sachsen-Anhalt); die + markieren die Lage der Grundwassermessstellen.

durch anaerobe Prozesse wie Sulfatreduktion geprägt (Fischer et al. 2004, Vieth et al. 2005).

Die hier beschriebenen Untersuchungen wurden im oberen, ca. 2–10 m mächtigen jungtertiären bis quartären Aquifer (GWL I) durchgeführt, der hauptsächlich aus Terrassenschottern der Weißen Elster besteht. Die vertikale Struktur der Kontaminationsfahne wurde im Bereich der Grundwassermessstelle Saf ZZ 23/00, die sich im nördlichen Abstrom des Kontaminationsherdes befindet, untersucht (Abb. 1).

Tiefenorientierte Grundwasserbeprobung

Die tiefenorientierte Grundwasserbeprobung wurde mithilfe eines Multilevel-Packersystems (MLPS) durchgeführt (Schirmer et al. 1995). Die Förderung des Grundwassers erfolgte dabei mit pneumatisch regulierbaren Miniatur-Doppelventil-Pumpen (Innovative Messtechnik Weiß, Deutschland), die eine gleichmäßige und blasenfreie Probennahme gewährleisteten. Bei geringer Förderleistung (ca. 100 ml/min) sowie einem Abstand zwischen den einzelnen Pumpen von etwa einem Meter werden bei der Entnahme der Grundwasserproben die vertikalen hydrogeochemischen Gradienten nicht durch Vermischungseffekte beeinflusst. Für die Erzeugung des benötigten Pumpendruckes wurde Stickstoff aus einer Druckflasche benutzt, um Sauerstoffeintrag während der Probennahme auszuschließen. Mit Ausnahme des Horizontes in 9,40 m Tiefe konnte aus allen verbleibenden 7 Tiefen das benötigte Probenvolumen für die Analysen entnommen werden (Abb. 2). Die Grundwasserproben wurden bis zur weiteren Analyse bei 4 °C kühl und dunkel gelagert.

Analytik der Grundwasserproben

Die Benzolkonzentrationen sind gaschromatographisch nach DIN 38407-F9-1 bestimmt worden. Die ionenchromatographische Analyse der Sulfat- und Nitrat-Konzentrationen basiert

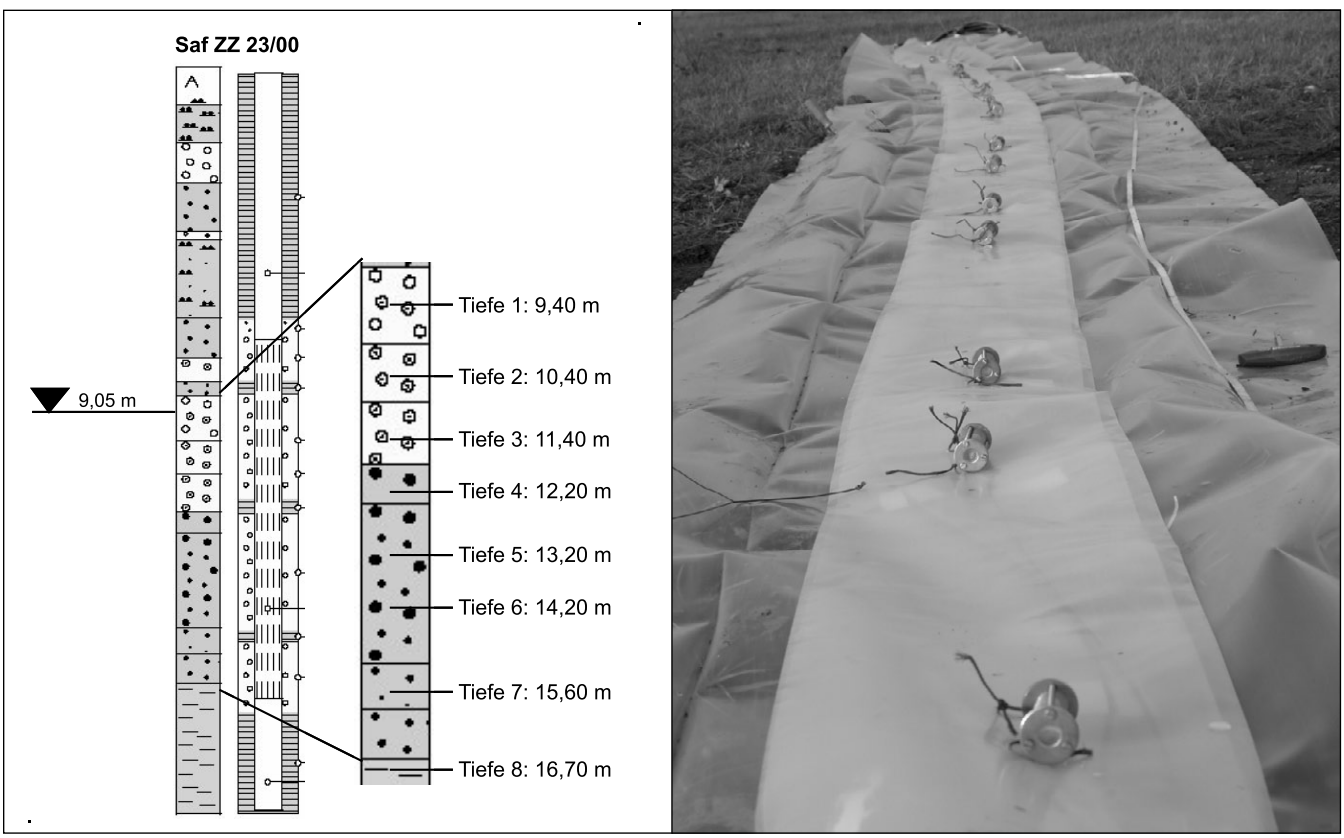

Abb. 2: Schichtenprofil und Ausbauplan der Grundwassermessstelle Saf ZZ 23/00. Tiefe 1 bis 8 bezeichnet die Position der Miniatur-Doppelventil-Pumpen und BACTRAPs (links). Foto des MLPS mit Edelstahlkäfigen für BACTRAPs vor dem Einbau in die Messstelle (rechts).

auf DIN EN ISO 10304-1 D19. Die Bestimmung der Konzentration des gelösten anorganischen Kohlenstoffs (DIC) erfolgte nach DEV D8, der Eisen(II)-Konzentrationen nach DIN 38406-E1-1 und der Eisen(III)- sowie Mangan-Konzentrationen nach DIN EN ISO 11885-E22.
Zur Bestimmung der Kohlenstoffisotopensignaturen des Benzols ($\delta^{13}C_{Benzol}$), des Methans ($\delta^{13}C_{CH4}$) und des DIC ($\delta^{13}C_{DIC}$) sind erprobte Methoden verwendet worden (Fischer et al. 2004, Vieth et al. 2005).

Herstellung der BACTRAPs

Bei der Herstellung der BACTRAPs dienten Bio-Sep®-Kugeln (K.L. Sublette; Universität Tulsa, USA) als Trägermaterial für die Testsubstanzen [^{12}C]- und [$^{13}C_6$]-Benzol und als Aufwuchskörper für die Mikroorganismen. Dabei handelt es sich um Kugeln mit 2 bis 3 Millimeter Durchmesser, bestehend aus 75 % pulverisierter Aktivkohle eingebettet in einer Matrix aus thermostabilem Nomex®. Die Oberfläche der Kugeln weist Poren mit einem Durchmesser von 2–10 µm auf. Bei einer Dichte von ca. 0,16 g/cm³ zeichnen sich die Kugeln besonders durch ihre hohe innere Oberfläche von > 600 m²/g Bio-Sep®-Material aus (White et al. 2003). Die Kugeln wurden zur Entfernung residualer organischer Kohlenstoffverbindungen bei 300 °C im Muffelofen für ca. 3 Stunden ausgeheizt.
Für die Herstellung der BACTRAPs ist ein Teflonrohr (VWR Darmstadt, Deutschland) mit 10 Millimeter Durchmesser in jeweils 40 Millimeter lange Stücke zerschnitten und anschließend perforiert worden, um einen Grundwasserdurchfluss gewährleisten zu können (Abb. 3). Pro BACTRAP wurden jeweils 0,4 g Bio-Sep® genutzt. Ein Glaswollestopfen am oberen und unteren Ende verhinderte das Herausfallen des Bio-Sep®-Materials.

Beladung der BACTRAPs

Die BACTRAPs wurden im Autoklav sterilisiert und hydratisiert. Im Anschluss erfolgte die Beladung der Mikrokosmen mit [^{12}C]- bzw. [$^{13}C_6$]-Benzol. BACTRAPs ohne Benzolaufschlag dienten als Kontrolle zur Analyse der mikrobiellen Besiedlung ohne zusätzliches Substrat.

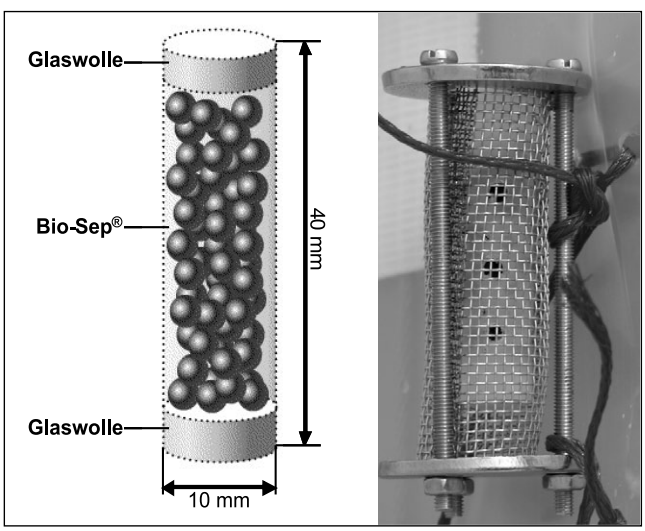

Abb. 3: Schematischer Aufbau eines BACTRAP (links) und Foto der Befestigung des Edelstahlkäfiges mit BACTRAP an der Packermembran (rechts).

Die BACTRAPs wurden in verschließbare Glasgefäße überführt und jeweils mit etwa 76 mg Benzol pro Gramm Bio-Sep® bei einem Unterdruck von ca. 60 mbar beladen. Um einen Gleichgewichtszustand in der Gasphase herzustellen und eine einheitliche Beladung der Aktivkohle mit dem Benzol gewährleisten zu können, verblieben die BACTRAPs zur Inkubation bei Raumtemperatur für mindestens 48 Stunden in dem verschlossenen Gefäß. Die Beladung ist mit einer Standardabweichung von ± 5 % reproduzierbar (Geyer et al. 2005). Die BACTRAPs wurden im Anschluss in einer Anaerobbox mit sterilem, anaeroben Wasser überschichtet, kurz evakuiert und dann unter anoxischer Atmosphäre entspannt. Dadurch füllte sich der Porenraum der Kugeln mit sauerstofffreiem Wasser und der Kontakt mit Luftsauerstoff beim Einbau der BACTRAPs wurde vermindert. Die BACTRAPs wurden in diesem Wasser bis zur Installation in den Brunnen gelagert.

Einbau in die Grundwassermessstelle

Die BACTRAPs wurden mittels MLPS in acht Tiefen der Messstelle Saf ZZ 23/00 (Abb. 2) eingebracht. Um eine gleichmäßige Durchströmung der BACTRAPs mit dem Grundwasser zu gewährleisten, wurden sie in Edelstahlkäfigen eingefasst (Abb. 3). Die Edelstahlkäfige dienten dabei als Abstandhalter um direkten Kontakt mit dem Pegelrohr sowie der Packermembran zu vermeiden.

Probenaufbereitung

Nach einer Expositionszeit von 51 Tagen wurden die Mikrokosmen aus der Messstelle entnommen und zur Konservierung bei –15 °C gelagert.
Die Extraktion der Fettsäuren erfolgte nach einer modifizierten Methode von Bligh & Dyer (1959). Dabei wurden 2 ml Methanol und Dichlormethan im Verhältnis 1:3 zugegeben. Für die Herstellung der Fettsäuremethylester (FAME) dienten 300 µl eines Derivatisierungsreagenz aus Chlortrimethylsilan und wasserfreiem Methanol im Verhältnis 1:8. Die Veresterung erfolgte bei einer Temperatur von 70 °C und einer Reaktionsdauer von zwei Stunden. Die FAME wurden in Hexan gelöst und anschließend mittels GC-MS bzw. GC-IRMS analysiert.

Identifikation der Fettsäuremethylester (FAME) mittels GC-MS

Die Bestimmung der FAME erfolgte mit einem Gaschromatographen (HP 6890 Series, Agilent Technology, USA) mit gekoppeltem Massenspektrometer (HP 5973 Mass Selective Detector, Agilent Technologie, USA). Zur gaschromatographischen Trennung der FAME wurde eine BPX 5-Säule (30m × 0,32 mm × 0,25 µm FD; Agilent Technologie, USA) eingesetzt. Das Temperaturprogramm beginnt bei 120 °C. Nach 4 Minuten steigt die Temperatur mit 4 °C/min auf 250 °C und schließlich mit 20 °C/min auf 300 °C, die abschließend 10 Minuten konstant gehalten wurden. Die FAME sind mithilfe des Bacterial Acid Methyl Ester-Standards (BAME, Sigma-Aldrich, Deutschland) durch den Vergleich der Retentionszeiten und Massenspektren identifiziert und auf ihre Reinheit geprüft worden. Die Fettsäuren werden nach folgender Nomenklatur benannt: A:BωC, wobei A die Anzahl der Kohlenstoffatome, B die Anzahl der Doppelbindungen und C die Entfernung der nächsten Doppelbindung vom aliphatischen Ende des Moleküls repräsentiert (ω-Nomenklatur). Durch die Vorsilben iso (i-) und anteiso (a-) wird die Position der Methylverzweigung gekennzeichnet.

Analyse der Kohlenstoffisotopenverhältnisse der FAME mittels GC-C-IRMS

Zur Analyse der Isotopenverhältnisse der FAME wurde ein Gas Chromatography-Combustion Isotope-Ratio-Monitoring Mass Spectrometer System (GC-C-IRMS) verwendet. Das System besteht aus einem Gaschromatograph (HP 6890 Series, Agilent Technology, USA), der an einen Verbrennungsofen, ein GC-C-III-Interface (Combustion III, ThermoFinnigan, Deutschland), eine Wasserfalle (Nafion®-Membran) und ein Massenspektrometer (IRMS MAT 252, ThermoFinnigan, Deutschland) gekoppelt ist. Eine Beschreibung zur verwendeten Gerätekonfiguration geben Richnow et al. (2003).

Zur Auftrennung der FAME wurde eine BPX 5-Säule (50 m × 0,32 mm × 0,5 µm FD; SGE GmbH, Deutschland) mit folgendem Temperaturprogramm verwendet: eine Minute 70 °C isotherm, 20 °C/min auf 170 °C, 2 °C/min auf 280 °C und mit 20 °C/min auf 300 °C, die für 5 Minuten isotherm gehalten wurden.

Berechnungen

Die Isotopensignaturen werden als δ-Notation [‰] relativ zu dem internationalen VPDB-Standard (Vienna PeeDee Belemnite mit $^{13}C/^{12}C = 0{,}0112372$) (Clark & Fritz 1997, Hoefs 1997) angegeben (Gl. 1).

$$[1] \quad \delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{Probe} - \left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}}{\left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}} \cdot 1000$$

Während der Methylierung der Fettsäuren (FA) zu Fettsäuremethylestern (FAME) wird ein zusätzliches Kohlenstoffatom in das Produkt eingefügt, sodass die ursprüngliche Isotopensignatur aller FA konstant verändert wird. Die gemessenen $\delta^{13}C$-Werte der FAME wurden deshalb nach der Gleichung 2 korrigiert, um die Isotopenverhältnisse der Fettsäuren ($\delta^{13}C_{FA}$) zu ermitteln (Abraham et al. 1998).

$$[2] \quad \delta^{13}C_{FA} = \frac{\left[(C_n + 1)\cdot\delta^{13}C_{FAME} - \delta^{13}C_{MeOH}\right]}{C_n}$$

C_n entspricht der Anzahl der Kohlenstoffatome in den Fettsäuren, $\delta^{13}C_{FAME}$ ist die Isotopensignatur der Fettsäuremethylester und $\delta^{13}C_{MeOH}$ die Isotopensignatur des für die Methylierung benutzten Methanols [–38,15 ‰].

Mit der Rayleigh-Gleichung (Gl. 3) wird die Beziehung zwischen der Konzentrationsänderung der mikrobiell umgesetzten Substanz in Verbindung mit einer Änderung der Isotopensignatur durch den Isotopenfraktionierungsfaktor α dargestellt (Hoefs 1997).

$$[3] \quad \frac{R_t}{R_0} = \left(\frac{c_t}{c_0}\right)^{\left(\frac{1}{\alpha}-1\right)}$$

R_0 und R_t geben die Isotopenzusammensetzung (Gl. 4), c_t und c_0 die Konzentration des Substrats zum Zeitpunkt t und 0 der mikrobiellen Umsetzung an.

$$[4] \quad \frac{R_t}{R_0} = \frac{\delta_t{}^{13}C + 1000}{\delta_0{}^{13}C + 1000}$$

Der prozentuale biologische Abbau der residualen Schadstofffraktion wird nach Gleichung 5 berechnet (Meckenstock et al. 2002). Dabei wird (c_t/c_0) durch Umstellen der Rayleigh-Gleichung ersetzt (Gl. 6), sodass der prozentuale biologische Abbau unter Verwendung von Isotopenwerten quantifiziert werden kann.

$$[5] \quad B[\%] = \left(1 - \frac{c_t}{c_0}\right)\cdot 100$$

$$[6] \quad B[\%] = \left[1 - \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\frac{1}{\alpha}-1}\right)}\right]\cdot 100$$

Die Veränderung der Kohlenstoffisotopensignatur und der Konzentration des gelösten anorganischen Kohlenstoffs (DIC) innerhalb einer Schadstofffahne dienen als biogeochemischer Indikator und können zur Abschätzung der Schadstoffmineralisierung nach Gleichung 7 verwendet werden (Fischer et al. 2004).

$$[7] \quad A\cdot\delta^{13}C_{DIC\,(GA)} + (1-A)\cdot\delta^{13}C_{DIC\,(Q)} = \delta^{13}C_{DIC\,(M)}$$

Dabei bezeichnet $\delta^{13}C_{DIC(GA)}$ das Kohlenstoffisotopenverhältnis des DIC im Grundwasseranstrom der Kontamination. $\delta^{13}C_{DIC(Q)}$ steht für das Kohlenstoffisotopenverhältnis des während der Schadstoffmineralisierung gebildeten DIC. Dabei wird angenommen, dass $\delta^{13}C_{DIC(Q)}$ die Isotopensignatur des nicht abgebauten Schadstoffs (Ausgangssubstrats) in der Quelle trägt (Hunkeler et al. 1999). $\delta^{13}C_{DIC(M)}$ entspricht dem $^{13}C/^{12}C$-Verhältnis des DIC, das innerhalb der Schadstofffahne gemessen wurde. A und (1–A) stellen die Mischungsanteile der beiden Ausgangsisotopenverhältnisse ($\delta^{13}C_{DIC(GA)}$ und $\delta^{13}C_{DIC(Q)}$) dar, aus denen $\delta^{13}C_{DIC(M)}$ resultiert. Anhand der DIC-Konzentration im Grundwasseranstrom der Schadstofffahne ($C_{DIC(GA)}$) und mithilfe von A kann auf die Menge des in der Schadstofffahne gebildeten DIC ($C_{DIC(B)}$) geschlossen werden (Gl. 8).

$$[8] \quad C_{DIC\,(B)} = C_{DIC\,(GA)}\,\frac{(1-A)}{A}$$

Schließlich ist eine Abschätzung der mineralisierten Schadstofffraktion ($C_{Schadstoff}$) anhand des gebildeten DIC ($C_{DIC(B)}$) möglich (Gl. 9).

$$[9] \quad C_{Schadstoff}\,[M] = \frac{C_{DIC(B)}}{Z_C}$$

Z_C bezeichnet die Anzahl der Kohlenstoffatome des Schadstoffmoleküls, die bei der Mineralisierung umgesetzt werden.

Ergebnisse

Die Messstelle Saf ZZ 23/00 im nordöstlichen Abstrom der Schadstofffahne (Abb. 1) wies in Monitoringkampagnen der letzten Jahre BTEX-Konzentrationen in einer Schwankungsbreite von ca. 40 bis 700 mg/l auf. Im Dezember 2004 wurde mit einem MLPS die vertikale hydrogeochemische Struktur der BTEX-Schadstofffahne untersucht und der In-situ-Benzolabbau anhand der Isotopensignatur des Benzols und DIC charakterisiert. Um den mikrobiellen Benzolabbau innerhalb der verschiedenen geochemischen Zonen untersuchen zu können, wurde parallel ein BACTRAP-Experiment in acht Tiefen durchgeführt.

Geochemie

Benzol stellt mit einem Anteil von mehr als 99 % die wesentliche BTEX-Komponente dar. Toluol, Ethylbenzol sowie die Xylole überschreiten als Summenparameter in keinem Beprobungshorizont Gesamtkonzentrationen von 2 mg/l (nicht dargestellt). Die Benzolfahne ist vertikal strukturiert (Abb. 4). Der maximale Benzolgehalt von > 6.400 µmol/l (> 500 mg/l) wurde in 14 m Tiefe bestimmt. Zum oberen und unteren Schadstofffahnenrand nehmen die Benzolkonzentrationen bis auf 415 bzw. 1.670 µmol/l kontinuierlich ab.

Die Isotopensignatur des Benzols weist in den mittleren Tiefen $\delta^{13}C$-Werte zwischen –28,0 ‰ und –29,0 ‰ auf (Abb. 4), die im Wesentlichen den Werten des Benzols im Schadenszentrum (–28,5 bis –29,5 ‰) entsprechen (Fischer et al. 2004, Vieth et al. 2005). In dieser Zone der Kontaminationsfahne kann daher über die Isotopenfraktionierungsmethode kein bzw. nur ein geringer biologischer Abbau von Benzol nachgewiesen werden (Tab. 1). Im oberen und unteren Bereich des Vertikalprofils der Messstelle Saf ZZ 23/00 ist eine ^{13}C-Anreicherung in der residualen Benzolfraktion um 3 bzw. 6 ‰ festzustellen, die offensichtlich auf Abbauprozesse hinweist. Eine Quantifizierung des prozentualen biologischen Schadstoffabbaus (B %) wurde unter Annahme sulfatreduzierender bzw. methanogener Bedingungen durchgeführt. Die Berechnung basiert auf den von Mancini et al. (2003) für den Benzolabbau bestimmten Fraktionierungsfaktoren (α), mit α_S von 1,0036 für sulfatreduzierende und α_M von 1,0019 für methanogene Abbaubedingungen. Der mikrobielle Benzolabbau unter der Verwendung der schweren Isotopensignatur der Quelle von –28,5 und dem höheren Isotopenfraktionierungsfaktor für sulfatreduzierende Bedingungen (1,0036) zeigt Abbaugrade bis zu 59 % am oberen und bis 83 % am unteren Fahnenrand (Tab. 1). Die Berechnung mit dem Fraktionierungsfaktor für methanogene Bedingungen (1,0019) unter Annahme einer relativ leichten Benzolquelle (–29,5 ‰) ergibt einen entsprechend höheren Abbau von 89 bis 98 % für die Fahnenränder. Mithilfe der hier berechneten Variabilität der Abbaugrade und den gemessenen Benzolkonzentrationen (C_t) in den einzelnen Tiefen der Messstelle Saf ZZ 23/00 konnte auf die theoretischen Schadstoffkonzentrationen der Kontaminationsquelle (C_0) zu Beginn eines biologischen Abbaus (t = 0) zurückgerechnet werden (Tab. 1). Die Berechnung des Benzolabbaus für sulfatreduzierende Bedingungen zeigte, dass diese theoretisch bestimmten Konzentrationen von > 10.000 µmol/l im unteren Aquiferbereich in etwa mit den Benzolkonzentrationen der Schadstoffquelle (>12.800 µmol/l) vergleichbar sind. Unter der Annahme methanogener Abbaubedingungen ergeben sich hypothetische Benzolkonzentrationen für die Schadstoffquelle von > 90.000 µmol, die die physikalische Löslichkeit des Benzols im Wasser überschreiten und daher unrealistisch sind. Das unterstreicht frühere Ergebnisse, nach denen die Methanogenese im Vergleich zur Sulfatreduktion am Standort keinen

Tab. 1: Berechnung des biologischen Abbaus [B %] für sulfatreduzierende (α_S = 1,0036) und methanogene Bedingungen (α_M = 1,0019) unter Berücksichtigung der Schwankungsbreite der Isotopensignatur von Benzol in der Quelle (R_0 = –28,5 ‰ bis –29,5 ‰)
[1] Berechnung des biologischen Abbaus für sulfatreduzierende Bedingungen unter Annahme von R_0 = –28,5 ‰
[2] Berechnung des biologischen Abbaus für methanogene Bedingungen unter Annahme von R_0 = –29,5 ‰
[3] Berechnung der Benzolkonzentration der Quelle auf Grundlage von B % (S)[1]
[4] Berechnung der Benzolkonzentration der Quelle auf Grundlage von B % (M)[2]

Tiefe [m]	C_{Benzol} [µmol/l]	$\delta^{13}C_{Benzol}$ [‰]	B % (S)[1] [%]	B % (M)[2] [%]	C_0 (S)[3] [µmol/l]	C_0 (M)[4] [µmol/l]
10,4	415	–25,4 ± 0,4	59	89	1.013	3.857
11,4	2.557	–28,2 ± 0,2	9	51	2.795	5.208
12,2	4.901	–28,0 ± 0,4	13	55	5.608	10.886
13,2	6.159	–28,6 ± 0,2	–4	37	5.916	9.825
14,2	6.419	–27,4 ± 0,2	26	68	8.725	19.743
15,6	1.840	–22,3 ± 0,2	83	98	10.871	91.177
16,7	1.668	–22,3 ± 0,4	83	98	9.916	83.647

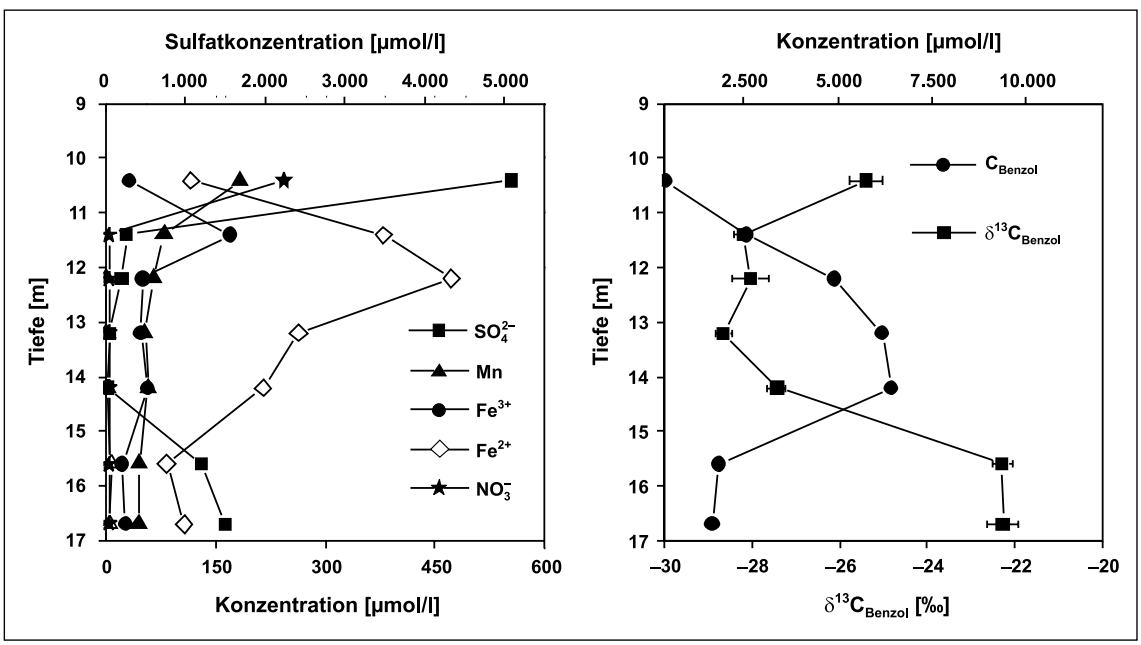

Abb. 4: Vertikale Verteilung der Eisen(II)-, Eisen(III)-, Nitrat-, Mangan-, Sulfat- (links) und der Benzolkonzentrationen sowie der Kohlenstoffisotopenverhältnisse des Benzols (rechts).

wesentlichen Anteil am Benzolabbau hat (Fischer et al. 2004). Unter sulfatreduzierenden Bedingungen ergibt sich ein Benzolabbau von bis zu 9.000 µmol/l (ca. 700 mg/l) auf dem Fließweg zwischen Quelle und Messpunkt im unteren Fahnenbereich, welcher aufgrund der hohen Benzolkonzentrationen im Schadenszentrum durchaus möglich sein könnte. Im oberen Rand der Fahne ergibt sich ein geringerer Benzolabbau zwischen 200 bis 700 µmol/l (16–55 mg/l).

In unbelasteten Bereichen des Aquifers werden Sulfatkonzentrationen von > 10.000 µmol/l nachgewiesen, sodass Sulfat den wichtigsten Elektronenakzeptor für mikrobielle Abbauprozesse darstellt (Fischer et al. 2004, Vieth et al. 2005). Im Vertikalprofil schwanken die Sulfatkonzentrationen zwischen ca. 20 µmol/l in einer Tiefe von 14 m und > 5.000 µmol/l am oberen Fahnenrand (Abb. 4). Verglichen mit unbelasteten Bereichen des Aquifers deutet die Abnahme des Sulfats infolge einer mikrobiellen Sulfatreduktion auf eine deutliche Sulfatsenke in der Fahne hin.

Der höchste Nitratgehalt von 244 µmol/l wurde am oberen Rand der Fahne im Übergangsbereich zur ungesättigten Zone festgestellt (Abb. 4). In tieferen Zonen konnte kein Nitrat nachgewiesen werden. Im Vergleich zum Sulfat steht für Abbauprozesse Nitrat als Elektronenakzeptor also nur in der oberen Zone des Aquifers in relativ geringen Konzentrationen zur Verfügung.

Die Konzentrationen von Mangan betragen etwa 180 µmol/l am oberen Rand der Fahne und reduzieren sich mit zunehmender Tiefe kontinuierlich auf Konzentrationen um 44 µmol/l (Abb. 4). Eine Manganreduktion könnte in der oberen Zone der Kontaminationsfahne einen Beitrag zum Schadstoffabbau liefern. Insgesamt sind die Mangankonzentrationen für einen signifikanten Schadstoffabbau jedoch zu gering.

Die Konzentrationen von Eisen(III) liegen im Bereich von ca. 20–60 µmol/l mit Ausnahme von ca. 170 µmol/l in einer Tiefe von 11,5 m. Die Eisen(II)-Werte schwanken zwischen etwa 80–470 µmol/l (Abb. 4). Erhöhte Fe(II)-Konzentrationen zwischen 11 und 14 m weisen auf eine Fe(II)-Mobilisierung hin, die durch Abbauprozesse verursacht sein könnte. Auffällig ist, dass die Bereiche erhöhter Fe(II)-Gehalte (11–14 m) durch die geringsten Sulfid-Konzentrationen gekennzeichnet sind. Diese liegen zwar generell bei ≤ 6 µmol/l (nicht dargestellt), aber die Fällung von Fe(II) durch Sulfid könnte zu einer Unterschätzung der Eisenreduktion beitragen. Gefälltes Eisensulfid verbleibt an der Aquifermatrix und kann im Grundwasser nicht quantitativ nachgewiesen werden, sodass der Anteil der Eisenreduktion anhand der Eisenmobilisierung kaum quantifizierbar ist. Es kann aber grundsätzlich davon ausgegangen werden, dass Eisen(III) am Standort keinen bedeutenden Elektronenakzeptor für Abbauprozesse darstellt (Wachter 2004).

Die Sauerstoffkonzentrationen liegen in der Messstelle mit Ausnahme des Übergangsbereichs zur ungesättigten Zone unterhalb von 40 µmol/l, sodass Sauerstoff als Elekronenakzeptor in tieferen Zonen der Fahne nicht zur Verfügung steht (nicht dargestellt).

Die DIC-Konzentrationen verhalten sich proportional zum Schadstoff (Abb. 5). Die höchsten Konzentrationen von > 17.000 µmolC/l wurden für den am stärksten kontaminierten Bereich zwischen 11 und 14 m registriert und nehmen am oberen und unteren Fahnenrand ab. Die maximal gemessenen DIC-Werte des Vertikalprofils entsprechen in der Größenordnung den DIC-Konzentrationen, die für die Hauptschadenszonen der BTEX-Fahne ermittelt wurden (Fischer et al. 2004). Im unteren Bereich der Fahne geht die DIC-Konzentration bis auf rund 1.300 µmolC/l zurück. Die Verteilung der verschiedenen Spezies des DIC im Grundwasser ist pH-Wert abhängig. Im Bereich der Schadstofffahne wurden pH-Werte von 6,5–7,0 bestimmt, sodass entsprechend des Kalk-Kohlensäure-Gleichgewichts davon auszugehen ist, dass Hydrogencarbonat die dominierende Spezies des DIC am Standort darstellt und Fällungsreaktionen ausgeschlossen werden können (Clark & Fritz 1997). Dieser Trend wird auch im Tiefenprofil der Messstelle Saf ZZ 23/00 beobachtet, wo neutrale pH-Werte von ca. 7,0 für alle Tiefen bestimmt wurden.

Unter der Annahme, dass die Sulfatreduktion der dominierende Prozess des mikrobiellen Schadstoffabbaus am Standort ist (Wachter 2004), kann folgende Abschätzung vorgenommen werden. Nach Wiedemeier et al. (1999) werden bei der Mineralisierung von 1.000 µmol Benzol unter der Bildung von 6.000 µmol CO_2 3.750 µmol Sulfat verbraucht. Eine mikrobielle Oxidation von

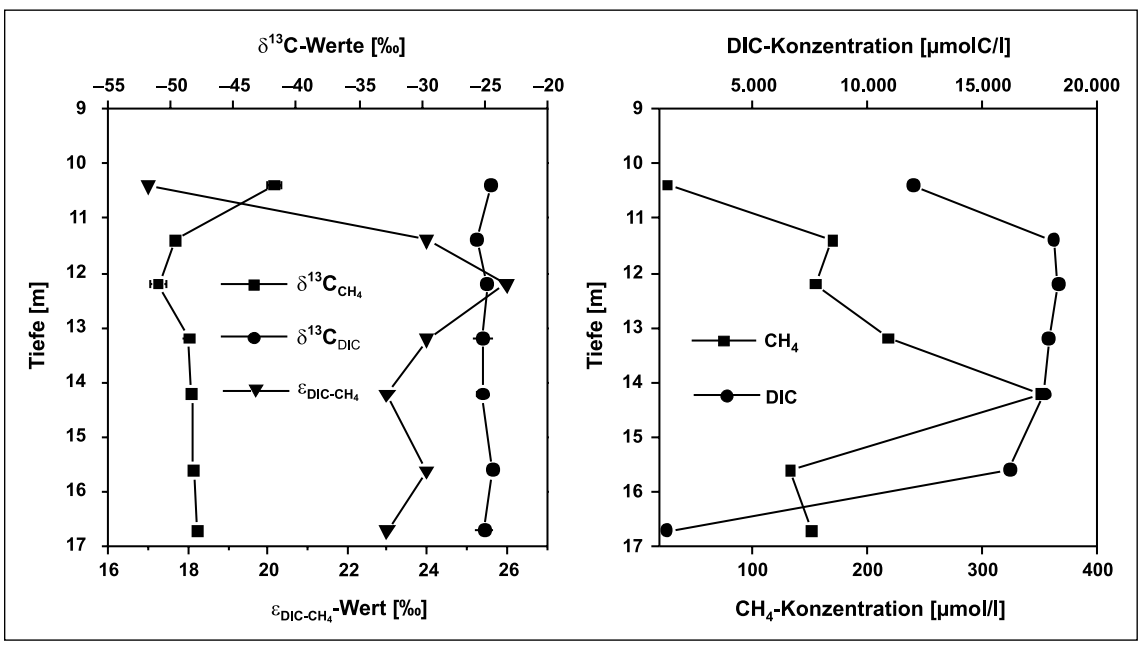

Abb. 5: Vertikale Verteilung der Kohlenstoffisotopenverhältnisse von Methan, DIC und den Anreicherungsfaktoren (links) sowie der Methan- und DIC-Konzentrationen (rechts) in der Messstelle Saf ZZ 23/00.

rund 1.300 µmol/l Benzol unter Bildung von etwa 8.000 µmolC/l DIC kann somit theoretisch eine Sulfatsenke von ca. 5.000 µmol/l bewirken, wenn der Aufbau zelleigener Biomasse unberücksichtigt bleibt. Verglichen mit der DIC-Konzentration von ca. 8.300 µmolC/l im Anstrom der BTEX-Fahne konnte eine maximale Konzentrationszunahme um ca. 10.000 µmolC/l im stark kontaminierten Bereich der Messstelle Saf ZZ 23/00 beobachtet werden. Aus der maximalen Differenz der DIC-Konzentrationen im Grundwasseranstrom und im Bereich der Messstelle Saf ZZ 23/00 berechnet sich ein theoretisches Abbaupotenzial von ca. 1.700 µmol/l Benzol. Aus den beiden Berechnungsansätzen resultiert ein Unterschied von etwa 400 µmol Benzol.

In kontaminierten Aquiferen gilt die Zunahme von Methan als ein Indiz für Biodegradation unter methanogenen Milieubedingungen (Wiedemeier et al. 1999). Die CH_4-Konzentrationen im Grundwasser können außerdem durch Methanoxidation beeinflusst werden (Breukelen & Griffioen 2004, Grossmann et al. 2002). Die Methanogenese führt zur Bildung von isotopisch leichtem Methan während die aerobe Methanoxidation mit einer Anreicherung schwerer Isotope verbunden ist. Um Methanbildungs- oder Abbauprozesse voneinander zu unterscheiden, kann der Anreicherungsfaktor $\varepsilon_{DIC\text{-}CH4}$, die Differenz zwischen der Kohlenstoffisotopensignatur des DIC und Methans, genutzt werden (Fischer et al. 2004). Geringe $\varepsilon_{DIC\text{-}CH4}$-Werte von < 20 ‰ gelten als Indiz für Methanoxidation, höhere Anreicherungsfaktoren deuten auf Methanbildung hin (acetoklastische Methanogenese 10–50 ‰; > 50 ‰ CO_2-Reduktion) (Fischer et al. 2004, Whiticar 1999). Die berechneten Anreicherungsfaktoren für das Tiefenprofil der Messstelle Saf ZZ 23/00 variieren von 17 bis 26 ‰ (Abb. 5). Im Beprobungshorizont bei 10,4 m wurden sowohl die geringsten Methankonzentrationen als auch die geringsten Anreicherungsfaktoren bestimmt, sodass im Übergang zur ungesättigten Zone des Aquifers Methanoxidation denkbar wäre. Mit zunehmender Tiefe trägt wahrscheinlich die Methanogenese zum Abbau der BTEX bei und führt zu einer Erhöhung der CH_4-Konzentration auf ca. 350 µmol/l, was etwa einem Abbau von 93 µmol (7 mg/l) Benzol entspricht (Wiedemeier et al. 1999). Wie anhand der Anreicherungsfaktoren abgeleitet werden kann, ist die Acetatgärung hierbei vermutlich der dominierende Methanbildungsprozess. Die relativ niedrigen Methankonzentrationen insgesamt zeigen jedoch, dass die Methanogenese keinen signifikanten Beitrag am Schadstoffabbau liefert.

Bei der vollständigen Mineralisation von Benzol entsteht Kohlendioxid, das die gleiche Isotopensignatur wie das Ausgangssubstrat trägt (Fischer et al. 2004, Hunkeler et al. 1999). Im Anstrom der Fahne liegt die natürliche DIC-Isotopensignatur im Mittel bei –19,5 ‰ (Fischer et al. 2004), im Bereich der Messstelle Saf ZZ 23/00 sind die $\delta^{13}C_{DIC}$-Werte hingegen durchschnittlich um ca. 5,5 ‰ leichter (–24,5 bis –25,6 ‰) und zeigen keine markanten Unterschiede über die Tiefe (Abb. 5). Werte dieser Größenordnung sind generell im stärker kontaminierten Bereich der BTEX-Fahne beobachtet worden (Fischer et al. 2004). Anhand dieser Veränderung des Isotopensignals über die Fließstrecke wurde die Umsetzung von mineralisiertem Benzol berechnet (Gl. 7–Gl. 9). Als Kohlenstoffisotopenverhältnis ($\delta^{13}C_{DIC(GA)}$) bzw. Konzentration ($C_{DIC(GA)}$) des DIC im Grundwasseranstrom wurde ein mittlerer Wert von –19,5 ‰ für $\delta^{13}C_{DIC(GA)}$ bzw. 8.300 µmolC/l für $C_{DIC(GA)}$ angenommen (Fischer et al. 2004). Das durchschnittliche Isotopenverhältnis des DIC im Profil der Messstelle Saf ZZ 23/00 ($\delta^{13}C_{DIC(M)}$) betrug ca. –25,0 ‰. Unter der Annahme identischer Isotopensignaturen des Benzols im Schadenszentrum und des DIC, das infolge einer Mineralisierung des Schadstoffs entsteht, wurde für die Quantifizierung ein $\delta^{13}C_{DIC(Q)}$-Wert von –28,5 ‰ angenommen, da im Fall einer vollständigen Mineralisierung die Isotopenbilanz geschlossen ist. Die Abschätzung ergibt für die Fließstrecke zwischen dem Zentrum der Schadstofffahne und der Messstelle Saf ZZ 23/00 eine Mineralisierung von etwa 2.160 µmol/l Benzol (169 mg/l).

Beim aeroben Benzolabbau kann eine Isotopenfraktionierung zwischen nicht abgebautem Benzol und gebildetem DIC von bis zu 4–6 ‰ auftreten (Hunkeler et al. 2001), wenn Biomasse gebildet wird oder der Abbau unvollständig ist. Die Fraktionierung zwischen Benzol und DIC beim anaeroben Abbau ist noch nicht untersucht. Würde eine Fraktionierung zwischen nicht abgebautem Benzol und gebildetem DIC von maximal 6 ‰ ($\delta^{13}C_{DIC(Q)}$ = –34,5 ‰) bei der Quantifizierung des anaeroben Benzolabbaus berücksichtigt werden, so verringert sich die Menge des mineralisierten Benzols (808 µmol/l bzw. 63 mg/l) erheblich. Die Abschätzung des Abbaus setzt voraus, dass die Quellen organischen Materials für das DIC isotopisch charakterisiert sind. Am Standort Zeitz steht vorherrschend Benzol zur Verfügung. Wenn andere Quellen wie natürlicher gelöster Kohlenstoff (DOC) oder andere Schadstoffe zur DIC-Bildung beitragen, ist eine Abschätzung schwierig. Es ist daher offensichtlich, dass die Abschätzung des Abbaupotenzials über eine Isotopenbilanz mit Unsicherheiten verbunden ist.

Ergebnisse der tiefenorientierten Untersuchungen mit den BACTRAPs

Die BACTRAPs mit und ohne Benzolbeaufschlagung (^{12}C-Benzol, ^{13}C-Benzol, Kontrolle) zeigen für alle Beprobungshorizonte vergleichbare Fettsäuremuster. Dominierende Fettsäuren sind C16:0 (> 40 %) und C18:0 (> 20 %) sowie die ungesättigten Fettsäuren C16:1 und C18:1 mit einem prozentualen Anteil von jeweils ca. 10 %. Untergeordnet, in geringen Konzentrationen (< 10 %), konnten die Fettsäuren C14:0, C15:0, i-C15:0, a-C15:0, C18:2 sowie eine weitere C18:1 Fettsäure mittels GC-MS identifiziert werden.

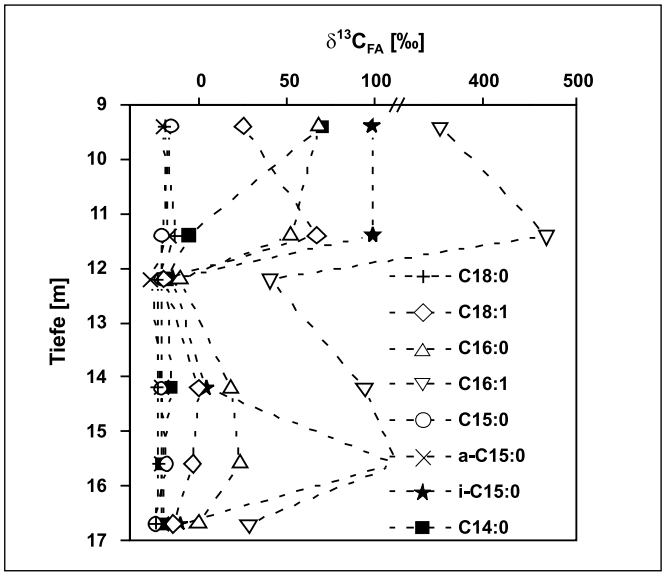

Abb. 6: Übersicht der Isotopensignaturen der Fettsäuren $\delta^{13}C_{FA}$ [‰] in den einzelnen Beprobungshorizonten. Die Abkürzungen und Nomenklatur der Fettsäuren sind im Kapitel Material und Methoden erklärt.

Da sich die Fettsäuremuster der mit Benzol beladenen und unbeladenen BACTRAPs nicht wesentlich voneinander unterscheiden, konnte weder ein Substrateinfluss noch eine Abhängigkeit des mikrobiellen Konsortiums vom geochemischen Milieu in den einzelnen Tiefen festgestellt werden. Die Fettsäuren liefern daher keinen Hinweis auf eine unterschiedliche Besiedlung der BACTRAPs in den verschiedenen Beprobungshorizonten. Verglichen mit einem BACTRAP-Experiment, das in einer Messstelle im weiter entfernten Grundwasserabstrom durchgeführt wurde (Geyer et al. 2005), sind die Fettsäuremuster im Bereich der Messstelle Saf ZZ 23/00 homogener und weniger komplex. Das lässt auf eine geringere Biodiversität auf dem BACTRAP schließen.

Die Kohlenstoffisotopensignaturen der Fettsäuren ($\delta^{13}C_{FA}$) aus dem Kontrollexperiment variieren zwischen –23 und –34 ‰. Die Isotopenanalysen der Proben aus dem Markierungsexperiment zeigten die höchsten ^{13}C-Anreicherungen (zwischen 28 und 468 ‰) für die C16:1 Fettsäure (Abb. 6). Eine etwas geringere Markierung wurde für die Fettsäuren i-C15:0, C14:0, C16:0 und C18:1 (–20 ‰ bis 145 ‰) bestimmt. Die signifikante ^{13}C-Anreicherung von Fettsäuren ist auf die Transformation von ^{13}C aus dem Benzol in die Biomasse zurückzuführen. Keine signifikante Transformation der ^{13}C-Markierung des Benzols wurde in die a-C15:0, C15:0 und C18:0 mit $\delta^{13}C_{FA}$-Werten von –14 bis –25 ‰ beobachtet (Abb. 6).

Die Isotopensignatur der Fettsäuren einer Spezies spiegelt, abgesehen von geringen Unterschieden, die auf Isotopenfraktionierungsprozessen beruhen, im wesentlichen das Isotopenverhältnis des Wachstumssubstrates wider (Abraham et al. 1998). Auch in Experimenten mit markierten Kohlenstoffsubstraten ist die Isotopensignatur bei PLFA verschiedener Kettenlänge etwa gleich (Pelz et al. 2001a) und auch kaum vom Wachstumsstadium beeinflusst (Abraham et al. 1998). Der Kohlenstoff wird also etwa gleichermaßen zum Aufbau von Membranlipiden verwendet. Die unterschiedlich starke Markierung zeigt daher die Besiedlung des BACTRAPs durch mikrobielle Konsortien, welche ^{13}C-Benzol unterschiedlich effektiv als Kohlenstoffquelle nutzen. Zum Beispiel scheinen Organismen, die unmarkierte C18:0, a-C15:0 oder C15:0 Fettsäuren besitzen, das markierte Benzol nicht oder nur kaum zu metabolisieren und bevorzugen andere Kohlenstoffquellen zur Synthese. Als zusätzliche Kohlenstoffquellen, die den Mikroorganismen zur Verfügung stehen können und die durch eine natürliche Isotopensignatur gekennzeichnet sind, kommen aromatische Kohlenwasserstoffe wie zum Beispiel BTEX aus der Kontamination sowie auch DOC in Frage.

Anhand der Intensität der ^{13}C-Anreicherung in den Fettsäuren konnten Zonen unterschiedlicher mikrobiologischer Aktivität identifiziert werden. Im obersten Bereich der untersuchten Messstelle (1. und 3. Tiefe) zeigt die isotopische Anreicherung in der C16:1 von > 350 ‰ demnach die größte mikrobielle Aktivität an. Im mittleren Tiefenintervall (12 m) wurden die geringsten Markierungen (40 ‰) für C16:1 nachgewiesen. Im unteren Bereich der Messstelle (14–15 m) zeigt die C16:1 mittlere ^{13}C-Anreicherung von ca. 94 ‰ (14,2 m) bzw. 188 ‰ (15,6 m). Die Ergebnisse verdeutlichen, dass nicht nur in den Randbereichen der Schadstofffahne sondern auch in den stark benzolbelasteten Zonen des Aquifers ein mikrobieller Benzolabbau stattfindet.

Diskussion

Geochemie

Die Abschätzung des Benzolabbaus anhand der Isotopensignaturen des Benzols bzw. DIC und Methan hat gezeigt, dass bevorzugt im oberen und hauptsächlich im unteren Randbereich der Fahne ein In-situ-Abbau stattfindet. Im Tiefenintervall von ca. 12–14 m geben die $\delta^{13}C_{Benzol}$-Werte keinen Hinweis auf mikrobiellen Abbau, da sie ungefähr das Isotopensignal des Quellbereichs (–28,5‰) tragen. Vermutlich ist im Bereich der sehr hohen BTEX-Konzentrationen der Anteil des mikrobiell abgebauten Benzols im Verhältnis zum nicht umgesetzten zu gering, um eine Veränderung der Isotopensignatur eindeutig nachweisen zu können.

Die konservative Abschätzung des Abbaus auf dem Fließweg zwischen Quelle und Messpunkt anhand der Isotopenmethode ergab für sulfatreduzierende Bedingungen einen Benzolabbau von bis zu 9.000 µmol/l (ca. 700 mg/l). Der gemittelte Abbau über alle Tiefen liegt bei ca. 3.000 µmol/l (ca. 230 mg/l). Mithilfe der Konzentrationen und Isotopensignaturen des DIC wurde ein Benzolabbau von etwa 2.160 µmol/l (ca. 169 mg/l) abgeschätzt. Aus der Elektronenbilanz über die Sulfatsenke ergab sich ein Abbaupotenzial von 1.700 µmol/l (ca. 133 mg/l) Benzol. Etwa 350 µmol/l (ca. 28 mg/l) Benzol könnten zusätzlich über methanogene Prozesse abgebaut worden sein. In der Summe errechnet sich ein möglicher Benzolabbau infolge Sulfatreduktion und Methanogenese von 2.050 µmol (ca. 160 mg). Dieser Wert entspricht etwa der Abschätzung der Mineralisation über das DIC. Die Quantifizierung des Abbaus anhand der Benzolisotope liegt etwa um ein Drittel höher aber in der gleichen Größenordnung. Die verschiedenen Berechnungsansätze liefern in der Größenordnung übereinstimmende Ergebnisse, sodass die Werte für den berechneten Benzolabbau realistisch erscheinen.

Eine Diskussion der Abweichungen der verschiedenen vorgestellten Ansätze zur Abschätzung des anaeroben Benzolabbaus gestaltet sich schwierig. Das Karbonatsystem (DIC) könnte durch Fällungen beeinflusst werden. Dies scheint aufgrund neutraler pH-Werte am Standort jedoch unwahrscheinlich zu sein. Ausgasungen in erheblichem Umfang sind nur im Bereich der Zonen mit direktem Kontakt zur ungesättigten Bodenzone zu erwarten und über die Mächtigkeit des gesamten Aquifers eher als gering anzusehen. In der Elektronenbilanz fehlt im Wesentlichen die Eisenmobilisierung, weil Fe(II) unter sulfatreduzierenden Bedingungen gefällt wird und für quantitative Abschätzungen nicht erfasst werden kann.

Die Beurteilung des mikrobiellen Schadstoffabbaus über die Methode der Isotopenfraktionierung gibt vermutlich eine realistische Größe für den Abbau zwischen Quelle und Messpunkt. Die Methode führt eher zu einer konservativen Abschätzung, wobei der mikrobielle Abbau nicht wesentlich überschätzt werden sollte. Mischungsprozesse auf dem Fließweg von Grundwasserströmen mit einerseits kaum und andererseits stark abgebauten Schadstoffmengen können zu einer Unterschätzung des tatsächlichen Abbaus führen (Fischer et al. 2004, Richnow et al. 2003). Zu einer Überschätzung des Abbaus führt die Isotopenmethode in der Regel nicht.

BACTRAP

Die Auswertung des BACTRAP-Experiments hat gezeigt, dass in allen untersuchten Tiefen ein Einbau von ^{13}C-Kohlenstoff in

die Fettsäuren erfolgte. Es konnte daher nachgewiesen werden, dass über den gesamten Bereich des untersuchten Vertikalprofils Benzolabbau stattfindet. Wie aufgrund der unterschiedlichen Intensität der Markierung abgeleitet werden kann, scheint der mikrobielle Benzolabbau offensichtlich in den Bereichen der maximalen Schadstoffbelastungen geringer als in den Randbereichen der Fahne zu sein. Da mit den BACTRAPs der mikrobielle Benzolabbau auch in stark kontaminierten Zonen nachgewiesen werden konnte, ist die Sensitivität dieses Testsystems wesentlich höher als beim Nachweis der Biodegradation über die Methode der Isotopenfraktionierung.

Die Quantifizierung des Benzolabbaus und Erstellung von Stoffumsatzbilanzen mit der Methode der BACTRAPs gestaltet sich problematisch. BACTRAPs verlieren unter vergleichbaren Bedingungen zu diesem Experiment etwa 80 bis 85 % der Menge des beaufschlagten Substrats (Benzol bzw. Toluol) (Geyer et al. 2005). Ein Teil des Substrats wird im Wasser gelöst und mit dem Grundwasserstrom abtransportiert. Dieser Verlust ist nur sehr schwer abschätzbar. Qualitativ konnte in anderen Multilevel-Versuchen eine Desorption des ^{13}C-markierten Substrats aus den BACTRAPs nachgewiesen werden. BACTRAPs mit nicht markierten Substraten, die in ca. 25 cm Entfernung zu ^{13}C-markierten BACTRAPs exponiert waren bzw. Grundwasserproben aus ähnlicher Tiefe zeigten eine ^{13}C-Anreicherung (unveröffentlichte Ergebnisse). Zukünftig sind Referenzversuche mit inerten, nicht abbaubaren Substraten zur Bestimmung der Desorptionskinetik, zur Berechnung des den Mikroorganismen tatsächlichen zur Verfügung stehenden markierten Substrats sowie zur Abschätzung von Verlusten nötig.

Ein weiterer Teil des Substrats wird offensichtlich mineralisiert und als CO_2 bzw. Methan aus dem BACTRAP in den Grundwasserstrom abgegeben und steht somit zur Quantifizierung einer geschlossenen Massenbilanz nicht zur Verfügung. Da Mikroorganismen in der Regel einen bestimmten Teil des Substrates, auf dem sie wachsen, zur Synthese eigener Biomasse verwenden, könnten zur Abschätzung der Schadstofftransformation Ertragskoeffizienten dienen. Ertragskoeffizienten beschreiben das Verhältnis zwischen Mineralisierung und Bildung von Biomasse und könnten in geschlossenen Modellsystemen bestimmt werden. Ex-situ-Referenzversuche im Labor sind allerdings schwierig, da anaerob Benzol abbauende Mikroorganismen gegenwärtig nur schwer kultivierbar sind. Alternativ könnte eine Abschätzung der Ertragskoeffizienten für die verschiedenen terminalen Elektronenakzeptoren mittels Referenzkulturen in Versuchen mit leichter abbaubaren Substraten wie Toluol oder Acetat bestimmt werden, die dann zunächst als Richtwerte für verschiedene biogeochemische Bedingungen zur Verfügung stehen.

Zukünftig soll anhand der Isotopensignatur der Anteil des in die Biomasse transformierten Substrats quantifiziert werden, sodass eine Abschätzung der Mineralisierung perspektivisch möglich scheint. Methoden zur Extraktion der gesamten Biomasse aus den In-situ-Mikrokosmen werden gegenwärtig entwickelt.

BACTRAPs stellen ein bedeutendes Testsystem zum direkten Nachweis der im Grundwasserleiter stattfindenden Biodegradation dar. Der entscheidende Vorteil des Testsystems ist, dass der Nachweis des mikrobiellen Schadstoffabbaus unter realen Aquiferbedingungen innerhalb nur weniger Monate erfolgen kann. Ein Nachteil zu Laborversuchen besteht derzeit in der eingeschränkten Möglichkeit zur Bilanzierung des Abbaus. Laborsysteme können geschlossene Stoffbilanzen einfach erfassen. Das ist, wie bereits dargestellt, mit In-situ-Mikrokosmen zurzeit nicht möglich. Da Testverfahren für Sedimente und Böden im Labor in der Regel nicht die Komplexität der Umweltbedingungen reproduzieren können, ist eine Übertragung der quantitativen Laborergebnisse auf den Aquifer ein kontrovers diskutiertes, ungelöstes Problem. Außerdem erfolgt der anaerobe Abbau meist sehr langsam, weshalb Laborverfahren häufig relativ aufwendig und zeitintensiv sind. Deshalb besitzen im Vergleich zu diesen herkömmlichen Systemen sowohl die Untersuchung der Isotopenfraktionierung als auch In-situ-Mikrokosmen mit isotopisch markierten Substanzen ein erhebliches Potenzial zur Charakterisierung des mikrobiellen In-situ-Schadstoffabbaus in kontaminierten Grundwasserleitern.

Zusammenfassung

Eine tiefenorientierte Untersuchung von Kontaminationsfahnen erlaubt die Analyse hydrogeochemischer Gradienten im Aquifer. Konzentrationen von Elektronenakzeptoren geben Aufschluss über vorherrschende biogeochemische Prozesse. Die vertikale Analyse der Kontaminationsfahne anhand der Konzentration und Isotopensignatur von Kontaminanten liefert qualitative und quantitative Indikatoren für den In-situ-Schadstoffabbau. Anhand der Methode der Isotopenfraktionierung konnten Abbauprozesse an den vertikalen Fahnenrändern des untersuchten Tiefenprofils analysiert werden. Eine messbare Veränderung der Isotopensignatur kann meist erst nach einem relativ hohen Schadstoffumsatz nachgewiesen werden. Bei geringem Abbau ist die Isotopenmethode relativ unempfindlich. Zusätzliche Untersuchungen mit den BACTRAPs konnten hier weiterhelfen, da mit dieser Methode der Schadstoffabbau mit einer höheren Empfindlichkeit nachgewiesen werden konnte. Mit der Isotopenmethode ist eine quantitative Abschätzung des Abbaus auf dem Fließweg zwischen Quelle und Messpunkt möglich. Der Einsatz von In-situ-Mikrokosmen mit isotopisch markierten Substanzen kann als ein empfindliches Testsystem für den eindeutigen Nachweis des Abbaus genutzt werden. Es werden qualitative Informationen zum Abbaupotenzial im Bereich der untersuchten Messstelle gewonnen. Verglichen mit Abbauexperimenten im Labor geben In-situ-Mikrokosmen direkte Aussagen zur Biodegradation im Grundwasserleiter. Schwierigkeiten bestehen derzeit noch in der quantitativen Interpretation der Daten. Die vertikale Analyse von geochemischen Gradienten und Isotopensignaturen von Schadstoffen, DIC sowie Methan verbunden mit dem Einsatz von In-situ-Mikrokosmen liefert Informationen zu biogeochemischen Abbauprozessen, die für einen Schadensfall relevant sind. Der Einsatz von MLPS in Verbindung mit modernen geochemischen und isotopenchemischen Verfahren ermöglicht somit mit vertretbarem Aufwand die Gewinnung entscheidender Informationen für die Planung von Natural Attenuation-Vorhaben bzw. für die Überwachung von aktiven Sanierungs- bzw. Enhanced Natural Attenuation-Verfahren.

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Anhang B

Nachweis des mikrobiellen Schadstoffabbaus in Grundwasserleitern (2006)

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Nachweis des mikrobiellen Schadstoffabbaus in Grundwasserleitern

Innovative Isotopenverfahren erfassen Abbauprozesse direkt im kontaminierten Grundwasser

Feldanwendung: Einbringen von Bactraps in die Grundwasserbrunnen einer Altlast

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Bei der Sanierung von Altlasten spielen natürliche Selbstreinigungsprozesse auch aus Kostengründen eine immer wichtigere Rolle. Voraussetzung für die Planung von Maßnahmen sind sichere Nachweise der mikrobiellen Abbauprozesse. Innovative Isotopenverfahren wie die Isotopenfraktionierung und *In-situ*-Mikrokosmen, die sich hinsichtlich Qualität und Sensitivität ergänzen, erfassen Abbauprozesse direkt im kontaminierten Grundwasserleiter.

Die Planung und der Erfolg von Grundwassersanierungsprojekten sind maßgeblich von der Erkundung des Selbstreinungspotenzials eines kontaminierten Standortes abhängig. Heute erlaubt die Anwendung innovativer Erkundungsverfahren oft wesentlich kostengünstigere Sicherungs- und Sanierungsmaßnahmen. Für moderne Sanierungskonzepte, wie sie bereits standardmäßig im Rahmen des „Monitored-Natural-Attenuation"-Ansatzes in den USA [1] durchgeführt werden, spielt vor allem der intrinsische mikrobielle Abbau aufgrund seiner nachhaltigen Schadstoffminderung eine wesentliche Rolle [2, 3]. Somit wird eine Charakterisierung der mikrobiellen Abbauleistung oder zumindest des Abbauvermögens für die zuverlässige Beurteilung eines kontaminierten Grundwasserleiters und die Prognose eines Schadensfalls unerlässlich. Intensive Forschungsaktivitäten im Rahmen des vom Bundesministerium für Bildung und Forschung (BMBF) geförderten Forschungsverbundes KORA (Kontrollierter natürlicher Rückhalt und Abbau von Schadstoffen bei der Sanierung kontaminierter Grundwässer und Böden) zeigen, dass innovative Erkundungsverfahren hierbei eine Schlüsselfunktion haben. Zum Nachweis der natürlichen mikrobiellen Selbstreinigung können moderne Isotopenverfahren eingesetzt werden, welche die Isotopenfraktionierung des Schadstoffs oder den Umsatz von isotopisch-markierten Zielsubstraten im Rahmen von *In-situ*-Mikrokosmenexperimenten nutzen.

Isotopenfraktionierung zur Kennzeichnung des *In-situ*-Schadstoffabbaus

Das Konzept zur Nutzung der Isotopenfraktionierung bei der Untersuchung des *In-situ*-Schadstoffabbaus wurde schon vor einigen Jahren in TerraTech vorgestellt [4]. Seitdem ist das Konzept stetig weiterentwickelt worden. Dadurch liegen reichhaltige Erfahrungen zur qualitativen und quantitativen Beurteilung des mikrobiellen Schadstoffabbaus vor (**Tabelle 1**).

Der mikrobielle Abbau eines Schadstoffes ist häufig mit einer Veränderung des Isotopenverhältnisses, sprich einer Isotopenfraktionierung, verbunden. Dabei werden Moleküle, die nur aus leichten Isotopen aufgebaut sind, meist schneller durch Mikroorganismen verwertet als solche, die ein schweres Isotop aufweisen. In der Regel kommt es bei der mikrobiellen Umsetzung zu einer relativen Anreicherung der schwereren Isotope (^{13}C, ^{2}H) innerhalb des nicht abgebauten Schadstoffes. Häufig ist davon auszugehen, dass abiotische Prozesse wie Sorption, Verdünnung und Verflüchtigung keinen oder nur einen vernachlässigbaren Einfluss auf das Isotopenverhältnis von Schadstoffen in einem kontaminierten Grundwasserleiter haben. Somit ist der Nachweis einer signifikanten Isotopenfraktionierung hauptsächlich auf den mikrobiellen Schadstoffabbau zurückzuführen [5, 6]. Sofern bei der Kohlenstoff- und/oder Wasserstoffisotopenfraktionierung signifikante Unterschiede für entsprechende Abbauwege eines Schadstoffes auftreten, kann anhand der Analyse der Kohlen- und Wasserstoffisotopenverhältnisse eine Charakterisierung des mikrobiellen Schadstoffumsatzes in einem Grundwasserleiter vorgenommen werden [7].

Das Ausmaß der Isotopenfraktionierung einer organischen Verbindung wird von deren Molekülgröße beeinflusst. Da meist nur an der Position, an der die Reaktion stattfindet, ein deutlicher Isotopeneffekt auftritt und der Rest der Verbindung in seinem Iso-

topenverhältnis gleich bleibt, wird die Isotopenfraktionierung durch den nicht reaktiven Teil des Moleküls abgeschwächt. Je mehr Atome eines Elementes ein Molekül aufbauen, desto geringer ist der messbare Isotopeneffekt. Große Moleküle weisen daher in der Regel eine geringere Veränderung des Isotopenverhältnisses beim mikrobiellen Abbau auf. Die Kohlenstoffisotopenfraktionierung ist bei Molekülen mit mehr als 12 Kohlenstoffatomen, wie z.B. bei vielen PAKs, kaum nachweisbar (Tabelle 1). Für niedermolekulare PAKs wie Naphthalin und Methylnaphthalin, gab die Veränderung der Isotopensignatur erst bei hohem mikrobiellen Umsatz eine Indikation auf mikrobiellen Abbau [8]. Somit ist der Nachweis der Biodegradation dieser Verbindungen anhand der Isotopenfraktionierung entsprechend unempfindlich. Dagegen kann der Nachweis des *In-situ*-Schadstoffabbaus für kleine Moleküle wie MTBE, BTEX sowie chlorierte Ethene und Monoaromaten (CKW) schon bei geringerem mikrobiellen Umsatz geführt werden.

Tabelle 1: Anwendbarkeit innovativer Isotopenmethoden zur Charakterisierung des mikrobiellen *In-situ*-Abbaus typischer Schadstoffe (verändert nach [19])

Gruppe	Schadstoff	Isotopenfraktionierung	BACTRAPS®
Schadstoffe >12 C-Atome			⩛
Benzinadditive			
MTBE	Methyl-tert-Butylether	☒●○	☒
TBA	*Tert*-butylalkohol	☒ ○	
Monozyklische Kohlenwasserstoffe (BTEX)			
	Benzol	☒●○	☒
	Toluol	☒●○	☒
	Ethylbenzol	☒●○	⩛
	m/p/o-Xylol	☒●○	⩛
Phenole			
	m/p-Cresol	☒●	
Polyzyklische Kohlenwasserstoffe (PAK)			
	Naphtalin	☒●	
	2-Methyl-Naphtalin	☒●	
	Phenanthren		⩛
	andere		⩛
Halogenierte aliphatische Kohlenwasserstoffe (CKW)			
PCE	Poly-Chlorethen	☒●	
TCE	Tri-Chlorethen	☒●○	
DCE	cis/trans-Di-Chlorethen	☒●○	
	1,2/1,1-Di-Chlorethen	☒●	
VC	Vinylchlorid	☒●○	
	1,2-Di-Chlorethan	☒●○	
	1,1,2-Tri-Chlorethan	☒●	
Halogenierte aromatische Kohlenwasserstoffe (CKW)			
MCB	Mono-Chlorbenzol	☒●	☒
TCB	1,2,4-Tri-Chlorbenzol	☒ ○	

☒ = Anwendung erfolgreich erprobt
● = für anoxische Verhältnisse
○ = für oxische Verhältnisse
⩛ = Anwendung in bestimmten Fällen
= derzeit keine Anwendung

Eine wichtige Voraussetzung zur Interpretation des mikrobiellen Schadstoffabbaus mit Hilfe der Isotopenfraktionierung ist eine hinreichend isotopisch-homogene Kontaminationsquelle. Die Isotopensignatur eines Schadstoffs kann je nach Hersteller variieren [9]. Im Falle von isotopisch-inhomogenen Quellen besteht die Gefahr einer Fehlinterpretation, weil die Änderung der Isotopenverhältnisse neben dem mikrobiellen Schadstoffabbau auch durch Mischung unterschiedlicher Quellisotopensignaturen verursacht werden kann. In der Praxis spielen isotopisch-inhomogene Schadstoffquellen selbst auf großen Standorten erfahrungsgemäß eine geringe Rolle.

Bei der konventionellen Grundwasserprobennahme können sich unterschiedliche Grundwasserströme mit verschiedenen residualen Schadstoffgehalten stark vermischen. Das sich ergebende Isotopensignal wird dann vom Grundwasserstrom mit der höheren Schadstoffkonzentration, d.h. mit geringem Abbau, bestimmt. Das Isotopensignal aus dem Grundwasserstrom mit hohem Abbau wird somit überlagert. Die Folge ist eine Abschwächung der Isotopenfraktionierung [10, 11]. Eine tiefenorientierte Probennahme mit Hilfe von Schlauch- oder Scheibenpackern vermag Mischungsvorgänge zu vermindern, und der mikrobielle Schadstoffabbau kann anhand der Analyse der Isotopenfraktionierung in einem vertikalen Profil genau erfasst werden.

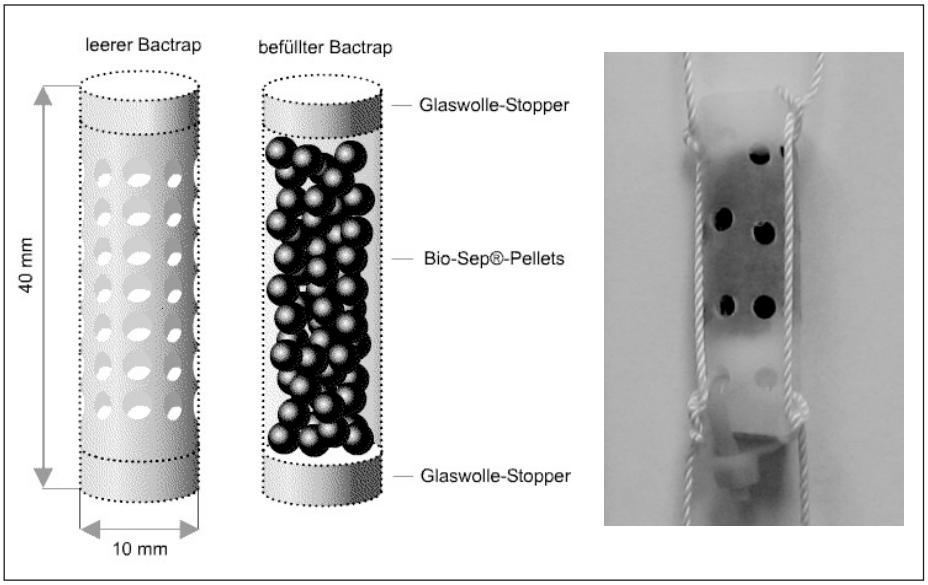
Zur quantitativen Bewertung des mikrobiellen Schadstoffabbaus wird die Veränderung des Isotopenverhältnisses zwischen der Kontaminationsquelle und den im Grundwasserabstrom befindlichen Messstellen verwendet. Die Methode erfasst somit die Schadstoffumsetzung über die jeweiligen Fließwege. Die Parametrisierung des mikrobiellen Abbaus erfolgt durch den Isotopenfraktionierungsfaktor (α) mithilfe der Rayleigh-Gleichung [5, 6]. α stellt den Zusammenhang zwischen der Änderung des Isotopenverhältnisses und der Konzentrationsabnahme während des mikrobiellen Abbaus her. Die schadstoffspezifischen Isotopenfraktionierungsfaktoren (α) können Übersichtsartikeln oder Datenbanken (www.isodetect.de) entnommen werden. Einen aktuellen Überblick über Studien zur Anwendung der Isotopenfraktionierung im Rahmen der Schadensfallbewertung geben Meckenstock et al. [5] und Schmidt et al. [6]. Dabei zeigt sich, dass das Konzept vor allem bei BTEX-, MTBE- und CKW-Schadensfällen eingesetzt wurde (z.B. [10, 7, 12]).

In-situ-Mikroskosmen zum Nachweis des mikrobiellen Schadstoffabbaus

Am Umweltforschungszentrum Leipzig-Halle (UFZ) wurde ein Verfahren zur empfindlichen Analyse der Biodegradation in Grundwasserleitern auf Grundlage von *In-situ*-Mikrokosmen (BACTRAP®) entwickelt. Diese können mit isotopisch-markierten Substanzen beladen werden. Der Abbau wird anhand der Isotopenmarkierung der Biomasse nachgewiesen. Radioaktiv markierte Substanzen (^{14}C) können in offenen Systemen auf Grund ökologischer Bedenken in der Regel nicht eingesetzt werden. Eine Alternative bieten organische Substanzen, die mit stabilen Isotopen (^{13}C; ^{15}N) markiert sind. Beim Abbau verwenden Mikroorganismen einen Teil des ^{13}C-markierten Kohlenstoffs zur Synthese von Biomasse. Analog können ^{15}N-markierte Substanzen zum Nachweis für deren Abbau eingesetzt werden.

Als Indikator für den mikrobiellen *In-situ*-Abbau der ^{13}C-markierten Testsubstanz kann die Transformation in die Fraktion der Phospholipid-Fettsäuren (*phospholipid-derived fatty acids*, PLFA) genutzt werden [13]. In früheren Untersuchungen wurden ^{13}C-markierte Substrate direkt in die Grundwasserleiter gegeben, um anhand der ^{13}C-Anreicherung in PLFA deren Metabolisierung nachzuweisen [14]. Diese Möglichkeit ist auf Grund des hohen Preises ^{13}C-markierter Substanzen in Verbindung mit dem hohen Mengeneinsatz in der routinemäßigen Anwendung kaum durchführbar.

Wesentlich geringere Mengen an ^{13}C-markiertem Substrat sind erforderlich, wenn eine vorherige Sorption an Trägerma-



Schematischer Aufbau (links) und Foto (rechts) eines Bactraps

terial erfolgt, dass in Form eines *In-situ*-Mikrokosmos (Bactrap) in Grundwasserbrunnen eingebracht werden kann. Als Trägermaterialien können Aktivkohlen, künstliche Polymere oder auch BIO-Sep®-Kugeln verwendet werden. So sind z.B. hochporöse Bio-Sep-Kugeln mit einer hohen inneren Oberfläche für die Sorption von Benzol und Toluol gut geeignet [15, 16]. Für die Herstellung der Bactraps wird ein perforiertes Teflonrohr mit den Bio-Sep-Kugeln oder einem anderen Trägermaterial befüllt, sterilisiert, hydratisiert und nachfolgend mit ^{13}C-markiertem Schadstoff beladen (siehe **Bild**). Bactraps mit unmarkiertem Substrat werden zur Kontrolle der natürlichen Isotopensignatur der Biomasse verwendet. Zum Nachweis des mikrobiellen Schadstoffabbaus wird die ^{13}C-Anreicherung in den Biomolekülen im Vergleich zu deren natürlichen Isotopenverhältnissen verwendet.

INFO Isodetect

Die im September 2005 entstandene Isodetect Umweltmonitoring GmbH in Neuherberg/Oberschleißheim ist eine gemeinsame Gründung von Mitarbeitern des Forschungszentrums für Umwelt und Gesundheit (GSF) in München und des Umweltforschungszentrums Leipzig-Halle (UFZ). Die Gründer, die sich seit den 90er-Jahren durch gemeinsame wissenschaftliche Arbeiten auf dem Gebiet der Mikrobiologie von Sedimenten kennen, haben nach der Patentanmeldung gemeinsamer Forschungsergebnisse zur Isotopenfraktionierung im Jahr 2000 die neuen Monitoringmethoden von Grundwasserkontaminationen zur Marktreife gebracht. Damit können jetzt aussagekräftige Nachweisverfahren für die Erkundung und Sanierung von Altlasten eingesetzt werden.
www.isodetect.de

Die Bactraps werden direkt im Grundwasserleiter über einen Zeitraum von sechs bis zwölf Wochen eingebracht. Kleine Mengen des beladenen Substrats werden während der Inkubation freigesetzt und können durch Mikroorganismen, die den Bactrap besiedeln, metabolisiert werden. In der Regel wird ein Bactrap mit etwa 50 bis 100 mg Substrat beladen. Nach etwa sechs Wochen Inkubation im Grundwasserleiter wurden im Fall von Toluol und Benzol noch 15 bis 18 % der ursprünglichen Beladung nachgewiesen [15, 16]. Auf den Bactraps bildet sich in der Regel ein Biofilm, aus dem die Fettsäuren der PLFA extrahiert und dann deren Isotopenverhältnisse bestimmt werden. Der Schadstoffabbau wird anhand einer deutlichen ^{13}C-Markierung der Fettsäuren nachgewiesen. Das Fettsäuremuster und die Anreicherung von ^{13}C-Kohlenstoff in einzelnen Fettsäuren kann Aufschluss über die Zusammensetzung mikrobieller Gemeinschaften geben [13].

In Feldversuchen konnte mit Hilfe von BACTRAPs der mikrobielle Abbau von Schadstoffen wie Benzol, Toluol, MTBE, Chlorbenzol und ausgewählter PAKs in kontaminierten Grundwasserleitern gezeigt werden [17, 15, 18, 16] (Tabelle 1). Bactrap-Systeme funktionieren jedoch nur, wenn Mikroorganismen die markierten Schadstoffe als Kohlenstoffquelle nutzen. Deshalb kann die Methode nicht zur Analyse des mikrobiellen CKW-Abbaus, der auf reduktiver Dehalogenierung beruht, angewendet werden. In diesem Fall nutzen die Mikroorganismen die CKWs nur zur Energiegewinnung und nicht als Kohlenstoffquelle.

Bactraps können zur vertikalen Untersuchung von Schadstofffahnen in Verbindung mit Scheiben- oder Schlauchpackersystemen eingesetzt werden. In der vertikalen Schichtung einer Schadstofffahne lassen sich Abbauprozesse entsprechend nutzbarer Elektronenakzeptoren nachweisen. Die vertikale Analyse eines BTEX-kontaminierten Grundwasserleiters mithilfe von Bactraps ergab unterschiedliche Abbaupotenziale in Abhängigkeit von Schadstoffkonzentrationen und bioverfügbarer Elektronenakzeptoren [16]. Die Identifikation abbauintensiver Zonen kann die Auswahl und Planung eines geeigneten Sanierungskonzeptes erleichtern.

Der Nachweis des mikrobiellen Schadstoffabbaus mit Hilfe von Bactraps bietet im Vergleich zu Labormikrokosmen einige Vorteile. Ein entscheidender Aspekt ist der Nachweis der *In-situ*-Biodegradation direkt im Grundwasserleiter. Darüber hinaus ist selbst bei schwer abbaubaren Schadstoffen ein Nachweis des Abbaus nach 30 bis 60 Tagen sogar unter anoxischen Bedingungen möglich. In dieser Zeitspanne ist der Nachweis im Labor meist nicht möglich. Experimente mit Labormikrokosmen sind oft zeitaufwändig, vor allem, wenn der anaerobe Schadstoffabbau eines kontaminierten Grundwasserleiters nachgestellt werden soll. Zudem sind die Bedingungen eines Grundwasserleiters selbst durch aufwändige Säulenversuche im Labor nur näherungsweise realisierbar. Aus diesen Gründen sind Labormikrokosmen in Routineuntersuchungen zur Analyse des aneroben *In-situ*-Schadstoffabbaus oft nur eingeschränkt geeignet. Labormikrokosmen mit Grundwasser oder Sedimentmaterial können aber auch interessante Informationen zum Abbaupotenzial liefern, die mit *In-situ*-Mikrokosmen nicht gewonnen werden können. So kann z.B. eine geschlossene Elektronenbilanz im Labormikrokosmos quantitativen Aufschluss über die Umsetzung des verwendeten Elektronenakzeptors geben.

Zusammenfassung

Zur Charakterisierung der Biodegradation von Schadstoffen in verunreinigten Grundwasserleitern können Untersuchungskonzepte auf Basis der Analyse der Isotopenfraktionierung oder von Bactrap-Systemen wertvolle Informationen liefern. Beide Konzepte erfassen Abbauprozesse direkt im kontaminierten Grundwasserleiter und ergänzen sich hinsichtlich ihrer Qualität und Sensitivität. Mit Hilfe der Isotopenfraktionierungsmethode kann der mikrobielle Schadstoffabbau und gegebenenfalls dessen initialer Schritt des Abbauweges erfasst und abgeschätzt werden. Bactraps liefern einen qualitativen Hinweis auf die *In-situ*-Biodegradation und können selbst kleine Abbauaktivitäten zweifelsfrei nachweisen. Die Anwendbarkeit beider Methoden ist unter Bezug auf bisher durchgeführte Studien in Tabelle 1 zusammengefasst. Grundsätzlich ist davon auszugehen, dass beide Konzepte zukünftig eine zunehmende Bedeutung bei der Planung und Durchführung von Sanierungsprojekten haben werden.

Ein wesentlicher Aspekt zur Implementierung innovativer Isotopenmethoden im Rahmen der Altlastenbewertung ist deren Zugänglichkeit für Behörden, Gutachter und Sanierungsfirmen. Aus diesem Grund hat sich im Jahr 2005 die Firma Isodetect, die innovative Isotopenmethoden zum Nachweis und Monitoring des mikrobiellen Schadstoffabbaus im Rahmen der wissenschaftlichen Dienstleistung anbietet, gegründet.

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Anhang C

In situ microcosms to evaluate natural attenuation potentials in contaminated aquifers (2006)

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In situ microcosms to evaluate natural attenuation potentials in contaminated aquifers

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Abstract

In situ microcosm (BACTRAP®) experiments with ^{13}C-labelled toluene and benzene were conducted to investigate the *in situ* biodegradation in contaminated aquifers at field sites with different geological and geochemical conditions. The transformation of the carbon (^{13}C), derived from labelled substrates, into fatty acids verified the assimilation of the pollutants with formation of biomass. This clearly demonstrated that the *in situ* microcosm system is a useful culture-independent method to investigate *in situ* biodegradation in the aquifer. In addition, metabolites such as benzylsuccinic acid were found on toluene amended BACTRAPs indicating that toluene was degraded anaerobically. This result corresponded to the geochemical conditions found at the field site and the approach enabled the analysis of the metabolic pathway governing *in situ* toluene biodegradation in the polluted aquifer.

Phospholipid fatty acids (PLFA) of living cells make up a significant part of the total lipid fatty acid (TLFA) fraction. Comparing BACTRAPs exposed at different geochemical zones of the aquifers, the fatty acid composition was found to be relatively similar indicating that the composition of the TLFA was of low taxonomic value and not sensitive enough for a community analysis. Therefore the composition of the microbial communities was analysed by genetic profiling and sequencing of partial 16S rRNA genes PCR-amplified from total DNA, extracted directly from the BACTRAPs. Sequences retrieved from the BACTRAPs indicated a dominance of not-yet cultivated bacteria, with several of them phylogenetically closely related to those with an iron and sulphate reducing capacity, typically found at BTEX and mineral oil polluted sites.

1. Introduction

The fate of pollutants in contaminated aquifers is mainly governed by microbial processes. Abiotic processes such as dispersion, dilution and volatilisation may contribute to a decrease in concentration but do not lead to a significant reduction of the

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overall amount of contaminants. Therefore, the evaluation of *in situ* biodegradation is crucial for the implementation of Natural Attenuation (NA) concepts in groundwater management strategies. Conventionally, complex calculations of decreasing contaminant concentrations related to the depletion of electron acceptors have been used to identify biodegradation processes. However, this method is difficult to apply in order to evaluate the fate of a single pollutant in contaminant mixtures on the field scale, particularly in the very heterogeneous natural environment with several competing electron acceptor–donor interactions. Except for the stable isotope fractionation technique (Meckenstock et al., 2004) and relatively labour intensive tracer experiments (Reusser and Field, 2002; Fischer et al., 2005) tools to quantify the actual *in situ* biodegradation of organic contaminants in the aquifer are scarce.

Culture-dependent laboratory microcosm studies are often used to investigate the potential of *in situ* biodegradation. However, this approach is not reliable because the majority of microorganisms have not been cultured yet and even the reproducible cultivation of anaerobic bacteria degrading typical contaminants such as BTEX and PAH is not an easy task. Laboratory microcosm studies as well as percolation column experiments, simulating the contaminant degradation under controlled water flow conditions, are time consuming and the conditions in the laboratory are certainly different to the actual natural environment complicating the interpretation of the results. Several limitations are related to these laboratory approaches particularly in providing reliable data to transfer the results to the actual ongoing *in situ* processes.

Recently, ^{13}C-labelled substrates in combination with ^{13}C-enrichment of PLFA were used to characterise bacterial toluene degradation in soil, sediment or aquifer microcosms (Hanson et al., 1999; Pelz et al., 2001a,b) and to trace the assimilation of toluene along a food chain (Mauclaire et al., 2003) providing useful information on carbon fluxes and metabolites. At the field scale, microbial incorporation of ^{13}C-labelled acetate into biomarker molecules like PLFA and DNA was successfully used to detect microbes which were suggested to be responsible for the reduction of uranium(VI) (Chang et al., 2005). To monitor the *in situ* dynamics of microbial communities in a BTEX polluted aquifer, a mesocosm study was designed (Hendrickx et al., 2005). In this study, uncontaminated aquifer material was incubated either in the uncontaminated area or nearby a contaminated area of the aquifer and it was shown that the bacterial communities were not identical in these two zones using molecular biological techniques.

To overcome the limitations of *ex situ* laboratory test systems and to improve the monitoring of *in situ* biodegradation, an *in situ* microcosm system (BACTRAP®) was developed that can be directly incubated within the groundwater monitoring wells (Geyer et al., 2005). The system consists of pellets containing activated carbon that can be loaded with ^{13}C-labelled contaminants. The pellets provide large amounts of interfacial area for colonisation or attached growth of degrading bacteria. If the indigenous bacteria colonise the BACTRAPs and consume the contaminants *in situ*, the ^{13}C-labelled carbon will be transformed into the biomass and can be traced within biomarker molecules such as fatty acids or nucleic acids.

In a first approach, BACTRAPs containing activated carbon beads were loaded with ^{13}C-labelled benzene and toluene prior to incubation within the anaerobic zone of a contaminated aquifer (Geyer et al., 2005; Stelzer et al., 2006). The pattern of fatty acids (FA) and the incorporation of ^{13}C into FA provided evidence for the *in situ* degradation of the test substrates and the metabolic transformation within a complex microbial community. In order to enable a more detailed characterisation of the bacterial community involved in biodegradation, we now analysed the microbial community colonising the *in situ* microcosms. For that reason the total DNA was directly extracted from the *in situ* microcosms and molecular tools were applied to study the microbial community structure. Partial sequences of the bacterial 16S rRNA genes, covering approximately 30% (400 base pairs) of the complete gene, were PCR-amplified from total DNA and the diversity of the amplified products was visualised by genetic profiling, using the single-strand conformation polymorphism technique (SSCP) (Schwieger and Tebbe, 1998; Dohrmann and Tebbe, 2004).

Because the adsorbing material of the BACTRAPs can enrich degradation metabolites we intended to identify the metabolic process and pathway governing the *in situ* biodegradation of toluene in the aquifer.

In this paper we present our recent progress with BACTRAP systems. We compare *in situ* microcosm experiments with different contaminants at different field sites. This *in situ* approach was used to monitor the biodegradation potential within different

geochemical zones of contaminated aquifers related to the vertical structure of a contamination plume. In addition, perspectives to use *in situ* microcosm experiments for microbial community structure analysis and for the identification of metabolic pathways are discussed.

2. Materials and methods

2.1. Chemicals

The chemicals and solvents were obtained in p.A. quality from Merck unless stated. [$^{13}C_6$] benzene, [^{13}C]-α-toluene and benzylsuccinate were obtained from Sigma–Aldrich (St. Louis, USA). [$^{13}C_7$] toluene was purchased from Chemotrade Leipzig (Germany). All the isotopically labelled compounds had a chemical and an isotope purity higher than 99%.

2.2. Field sites

Zeitz (Saxony-Anhalt, Germany): Benzene, toluene, ethylbenzene and *o*,*m*,*p*-xylene (BTEX) contaminated aquifer is located in the area of a former hydrogenation plant close to the city of Zeitz, Germany (Fig. 1). A detailed description of the site hydrogeology and hydrochemical conditions of the contamination plume is given in previous studies (Fischer et al., 2004; Vieth et al., 2005).

The BACTRAP experiments were performed in a multilevel approach in the upper aquifer to investigate different geochemical zones of the vertical BTEX plume. The thickness of the upper aquifer varies between 4 and 6 m. In the source area total BTEX concentrations exceeded 900 mg l^{-1} (Fig. 1). Benzene and toluene were present in concentrations up to 850 mg l^{-1} and 50 mg l^{-1}, respectively. Ethylbenzene and xylenes were typically present in concentrations lower than 3 mg l^{-1}.

At the test site the predominant electron acceptor used for biodegradation was sulphate (Vieth et al., 2005). Active sulphate reduction in the course of the plume was evident by an enrichment of ^{34}S-sulphate (Fischer et al., 2004). In addition, methanogenesis had a minor impact on microbial BTEX-transformation in the BTEX-plume but a more significant impact in the source area (Fischer et al., 2005). Other electron acceptors like oxygen, nitrate and iron played a minor role for the overall biodegradation processes at the site (Dethlefsen et al., 2004; Fischer et al., 2004; Vieth et al., 2005).

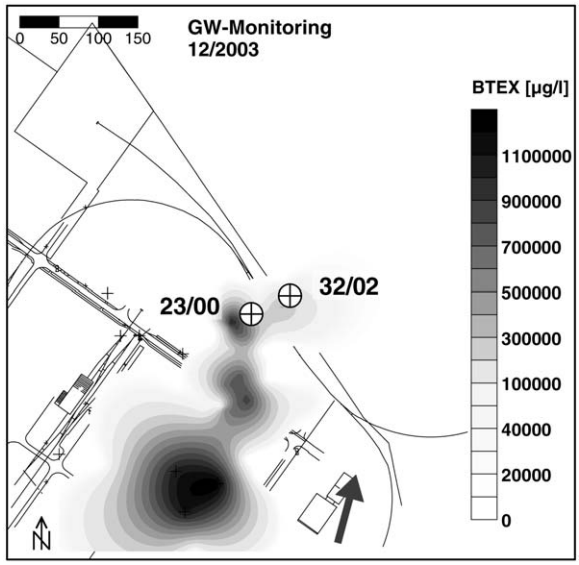

Fig. 1. Concentration of BTEX [µg l^{-1}] in the upper aquifer in the area of the former hydrogenation plant in Zeitz (Saxony-Anhalt, Germany). The locations of the *in situ* microcosm experiments are indicated. At well 23/00 the multilevel experiment was carried out and well 32/00 was used for the in situ microcosm study by Geyer et al. (2005). The arrow depicts the general groundwater flow direction.

Gneisenau (Dortmund, Germany): The anaerobic tar oil and BTEX contaminated aquifer is located at the former coking plant Gneisenau in the vicinity of a coal mine in the Ruhr area. The hydrological situation is characterised by two aquifers separated by erratic marls and tills. The hydraulic conductivity of the upper porous Quaternary aquifer is relatively low at the basis (k_f of about 10^{-9} m s^{-1}) but is significantly higher in sandy layers ($k_f = 10^{-6}$ to 10^{-4} m s^{-1}). The water table is located 5–20 m below ground. The lower aquifer is a confined fractured Cretaceous aquifer. Both aquifers are contaminated by BTEX, PAH and other contaminants typical for coking plants. The upper Quaternary aquifer was mainly contaminated by BTEX (up to 1.5 mg l^{-1}) and naphthalene (up to 750 µg l^{-1}), whereas in the deeper Cretaceous aquifer mainly benzene (up to 45 mg l^{-1}) was found. Oxygen concentrations were always below 1 mg l^{-1}. The isotope enrichment of ^{34}S-sulphate in most contaminated areas provided evidence for sulphate reduction (Pfeifer, 2004). Sulphate, with concentrations up to 1660 mg l^{-1}, is likely the most important terminal electron acceptor at this site. Geochemical investigations suggested that methanogenesis as well as nitrate, iron and manganese reduction are probably

less important for microbial processes in the subsurface (Pfeifer et al., 2003). The presence of benzylsuccinic acid, a typical metabolite of toluene, provided further evidence that anaerobic degradation processes are predominant at the site (Pfeifer, 2004).

2.3. Multi level packer systems used for depth discrete experiments

A multi level packer system (MLPS) was used for depth discrete sampling in Zeitz. The MLPS is a water-filled sock tube inserted into the well, which allows the separation of different sampling systems from each other at different depths. Small submersible pumps allowed depth-specific sampling of ground water without cross-currents. A detailed description of the MLPS is given by Schirmer et al. (1995).

The depth discrete investigation of geochemical and isotope parameters in Zeitz at well 23/00 were previously described by Stelzer et al. (2006). The position of BACTRAP systems, water table and geological information of well 23/00 are shown in Figs. 1 and 2.

2.4. Preparation and incubation of in situ microcosms (BACTRAP)

Bio-Sep® beads (K. Sublette, University of Tulsa, Tulsa, USA) were loaded with contaminants as carbon substrate for microorganisms. The spherical beads, 2–3 mm in diameter, consist of powdered activated carbon (PAC) incorporated within an aramid polymer matrix (Nomex®). The beads have an internal porosity of 75%, an internal surface area greater than 600 $m^2 g^{-1}$, and outer pores of 1–10 μm (Peacock et al., 2004; White et al., 2003). The beads were heated at 300 °C for 4 h to remove organic residues and then 0.2–0.4 g of beads were placed in perforated Teflon® tubes. Glass wool was used as a plug to keep the beads inside the Teflon® tubes. The filled Teflon® tubes were autoclaved at 121 °C for sterilization and re-hydration of the beads. The microcosms were loaded with the substrate (benzene or toluene) via gas phase under reduced pressure as described previously (Geyer et al., 2005). The substrate and the microcosms were placed in small glass containers which were evacuated at 60 mbar. To allow uniform adsorption of the contaminants the Bio-Sep® beads were incu-

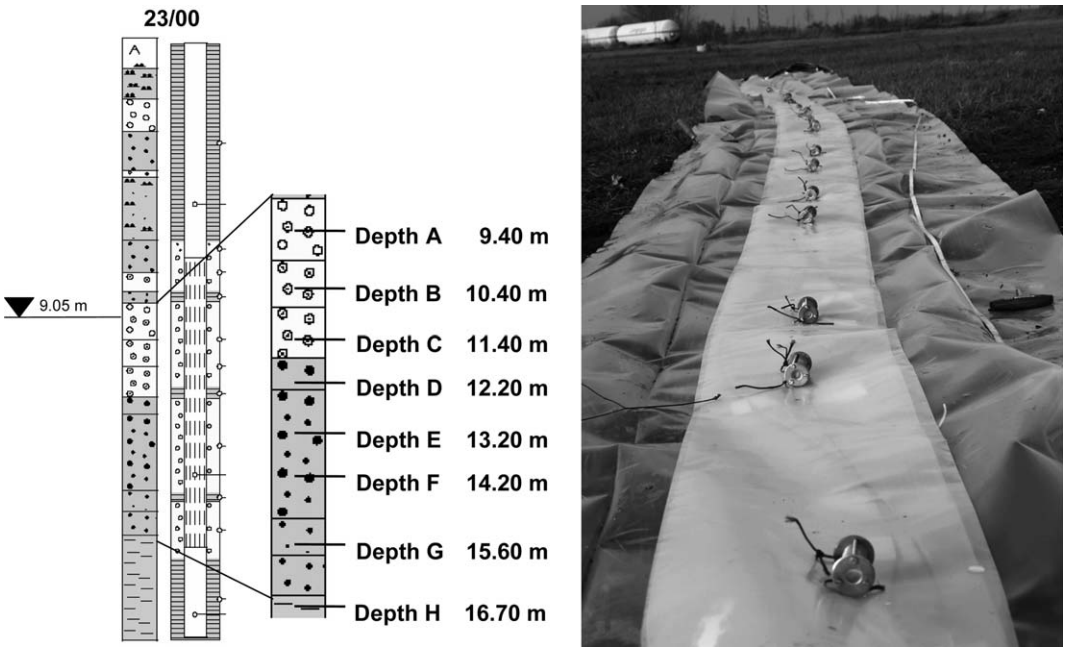

Fig. 2. Left: Geological profile at monitoring well 23/00 (Zeitz, Germany). The location of the filter screen is illustrated. The groundwater table was located at 9.05 m below ground. *In situ* microcosms were installed at A, C, D, F, G, H and samples for geochemical analyses were taken from all depths except A. Right: The picture shows the MLPS with stainless steel containers for *in situ* microcosms before subjection into the well.

bated for at least 48 h. Contrary to the previous method, the vacuum was then released by filling oxygen free water into the glass container and the BACTRAPs were stored under anoxic water until deployment in the monitoring wells. The oxygen free water was used to fill the free pore space of the beads due to the vacuum release to keep the *in situ* microcosm system anoxic. The BACTRAPs for the multi level experiment in Zeitz were loaded with [$^{13}C_6$] benzene to a concentration of about 76 mg g^{-1} and for the metabolite experiment with [^{13}C]-α-toluene to a concentration of about 50 mg g^{-1}. The BACTRAPs deployed in Gneisenau were loaded with uniformly labelled [$^{13}C_6$] benzene and [$^{13}C_7$] toluene to a concentration of 500 mg g^{-1}. In all experiments, BACTRAPs were loaded with non-labelled benzene or toluene in similar concentration for control experiments. Material from these control experiments was used for molecular biological studies and to investigate the isotope composition of fatty acids at natural abundance.

The microcosms were deployed with the MLPS or in monitoring wells over a time period of 51–100 days. In the MLPS experiments the microcosms were placed in stainless steel cages as a spacer to ensure a permanent flow of groundwater through the *in situ* microcosms and to avoid contamination with biological material by direct contact with the packer membrane or the filter screen of the well.

2.5. Extraction and derivatisation of fatty acids

The Bio-Sep® beads were extracted using a dichloromethane–methanol–water mixture as solvent modified from Bligh and Dyer (1959). After phase separation the dichloromethane phase containing the fatty acids (FA) was evaporated to dryness and derivatised using a trimethylchlorosilane (TMCS) methanol mixture (1:8; v:v) as reactant for 2 h at 70 °C to obtain fatty acid methyl esters (FAME) (Thiel et al., 2001). After evaporation to complete dryness the FAME fraction was dissolved in *n*-hexane for subsequent analysis by gas chromatography–mass spectrometry (GC–MS) and gas chromatography–combustion–isotope-ratio-monitoring-mass-spectrometer system (GC–C–IRMS).

2.6. Analysis

GC–MS: For identification and structural characterisation by GC–MS a Hewlett–Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent, Palo Alto, USA) was used. The carboxylic acid fraction was separated on a BPX-5 column (30 m × 0.32 mm × 0.25 μm) (SGE, Darmstadt, Germany) with a temperature program of 120 °C initial temperature for 4 min, heat at 4 °C min^{-1} to 250 °C, heat at 20 °C min^{-1} to 300 °C, and hold for 10 min. FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Sigma–Aldrich, Germany). The fatty acids are designated in the form of $A{:}B\omega C$ where A is the number of carbon atoms, B is the number of double bonds and C is the distance of the closest double bond from the aliphatic end of the molecule (unsaturation, ω-nomenclature). The prefix *i* (*iso*) and *a* (*anteiso*) refer to methyl branching. The benzylsuccinic acid methyl ester was characterised by co-injection and comparison of mass spectra of the authentic reference compound which was derivatised as described above.

GC–C–IRMS: The carbon isotope composition of the carboxylic acid fractions was analysed using a GC–C–IRMS. The system consists of a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany) as described previously (Richnow et al., 2003a). A BPX-5 column (50 m × 0.32 mm × 0.5 μm) (SGE, Darmstadt, Germany) was used for chromatographic separation of fatty acid methyl esters with helium as carrier gas at a flow rate of 1.5 ml min^{-1} and a temperature program with initial temperature of 60 °C for 2 min, heat at 20 °C min^{-1} to 120 °C, heat at 2 °C min^{-1} to 300 °C, and hold for 20 min (Miltner et al., 2004). The analysis of BTEX (Vieth et al., 2005) on the same system was described previously.

The carbon isotope ratio of fatty acids is reported in δ-notation (per mil) relative to the Vienna Pee Dee Belemnite standard (V-PDB) with known isotopic composition (Anonymus, 1995). The isotope composition of highly ^{13}C-enriched fatty acids could not be determined accurately. The instrument was calibrated to about 1000 δ units. Higher values are outside the calibration range of the instrument and the uncertainty increases with higher enrichments. Therefore, positive isotope compositions of fatty acids were only reported with the first 2 decimal places of the value.

2.7. Molecular biological analysis

For each sample, a total of 50 Bio-Sep® beads were transferred into 15 ml Falcon tubes containing 12 ml of TES buffer (50 mM $NaCl_2$, 10 mM Na_2EDTA, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride [Tris–HCl], pH 8.0) with 1% sodium dodecylsulphate (SDS). Five cycles of freeze–thaw lysis were performed, each with 5 min in liquid nitrogen (−196 °C) and 5 min at 65 °C in a shaking water bath, each cycle was interrupted by 10 s of vigorous vortexing. DNA was then extracted with phenol–chloroform, as described elsewhere (Schwieger and Tebbe, 1998, 2000). The isopropanol precipitated and purified DNA of each tube was resuspended in a total of 40 µl of 10 mM Tris in 10 µl aliquots. Aliquots were kept at 4 °C for further analysis or stored at −20 °C.

Partial 16S rRNA genes were amplified from total DNA by PCR, using primers Com1 and Com2ph (Schmalenberger et al., 2001). The PCR products were converted to single strands by λ-exonuclease digestion, following the protocol of Schwieger and Tebbe (1998). Genetic profiles of each sample were generated by single strand conformation polymorphism (SSCP) on non-denaturing polyacrylamide gels, as described in detail elsewhere (Tebbe et al., 2001; Dohrmann and Tebbe, 2004). Single bands were selected for further DNA-sequencing after PCR-amplification and cloning in *Escherichia coli*. Sequencing is described in the same references (Peters et al., 2000; Tebbe et al., 2001; Dohrmann and Tebbe, 2004). Consensus sequences were compared to database sequences using the FASTA tool provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta/). DNA-sequences of this study have been deposited in the GenBank database under the Accession Nos. AM26926–AM261950. Phylogenetic analyses were conducted using the ARB software package (Ludwig et al., 2004), as described elsewhere (Dohrmann and Tebbe, 2005).

3. Results and discussion

3.1. Zeitz

In order to test the BACTRAP concept, the BTEX contaminated aquifer in Zeitz (Germany) was chosen as a model site, because detailed information was available about the contamination plume and the geochemical zones from previous studies (Vieth et al., 2001, 2005; Fischer et al., 2004, 2005). At well 32/02 (Fig. 1), located downstream of the contamination source and characterised by moderate benzene concentrations of approximately 44 mg l^{-1} in 2002, *in situ* microcosms supplemented with ^{13}C-labelled toluene and benzene provided evidence for the *in situ* degradation of these substances, as described in detail elsewhere (Geyer et al., 2005).

For further BACTRAP studies we selected well 23/00 (Figs. 1 and 2) because of generally higher concentrations of contaminants and clear indication of microbial sulphate reduction as the predominant terminal electron accepting process by sulphur isotope analysis (Fischer et al., 2004).

A depth specific MLPS approach was chosen to investigate different geochemical zones of the aquifer within only one monitoring well (Fig. 2). The benzene plume showed a vertical structure at this monitoring well (Fig. 3). The benzene concentration increased from 415 µmol l^{-1} at 10.4 m below the surface, which was located near the water table at 9.05 m, to a maximum of more than 6000 µmol l^{-1} in the depth interval between 13 and 14 m. Towards the aquitard, located at about 17 m, the benzene concentration decreased to about 1700 µmol l^{-1} at 16.7 m depth.

In uncontaminated zones of the aquifer upstream from the plume, the concentrations of nitrate were relatively low (<100 µmol) compared to sulphate concentrations (>10 mmol) suggesting that nitrate was not a dominant electron acceptor in this aquifer. At the observation well manganese and nitrate were only present in the upper zone of the aquifer in elevated concentrations (Fig. 3). The low total manganese concentrations indicated that manganese mobilisation by Mn(IV) reduction was not an important electron accepting process at the field site. Thus nitrate and manganese may act as potential electron acceptors at the water table close to the capillary fringe, where also some oxygen may enter from the gaseous phase of the soil into the aquifer system. Ferrous iron mobilisation suggested that ferric iron was a potential electron acceptor. However, the concentration of ferrous iron may not show the true extent of iron reduction, because ferrous iron can be precipitated in the presence of H_2S. However, as discussed elsewhere iron reduction is not an important terminal electron accepting process in this aquifer (Dethlefsen et al., 2004). Sulphate concentrations >10 mmol l^{-1} are typical for uncontaminated parts of the aquifer (Fischer et al., 2004) and in the upper part of the vertical

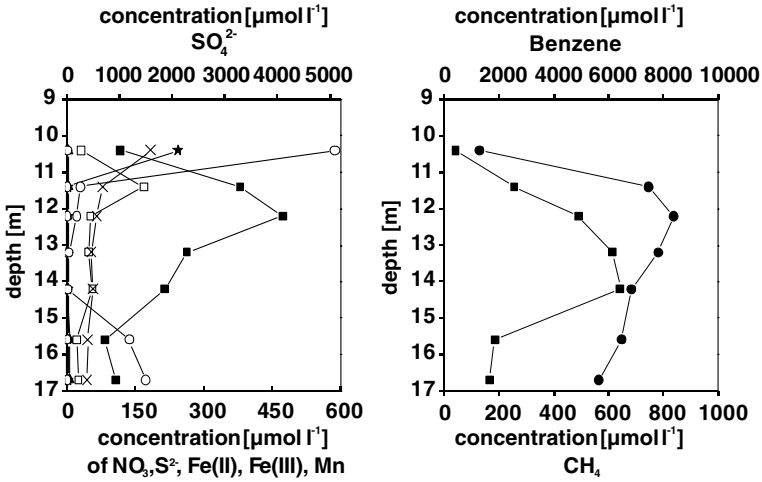

Fig. 3. Left: Concentration of sulphate (○), nitrate (*), sulphide (▲), ferrous iron (■), ferric iron (□) and manganese (×) in [µmol l^{-1}]. Right: benzene (■) and methane (●) concentration in [µmol l^{-1}] at Zeitz (Germany) monitoring well 23/00.

profile sulphate concentrations up to 5 mmol l^{-1} were found. The depletion of sulphate within the plume to concentrations of less than 0.2 mmol l^{-1} between 11.5 and 14.5 m suggested intensive microbial sulphate reduction although the development of sulphide was low. Sulphide may be precipitated by iron, which emphasises the difficulties to trace the degradation of contaminants by electron donor acceptor balances. Thus, sulphate was presumably the predominant electron acceptor.

Fatty acid composition: The fatty acid patterns extracted from the BACTRAPs were dominated by hexadecanoic (C16:0) and octadecanoic (C18:0) acids. Unsaturated hexadecenoic (C16:1) and octadecenoic (C18:1) acids were present in lower concentrations (Fig. 4). Other linear saturated fatty acids like tetra (C14:0), penta- (C15:0) and heptadecanoic (C17:0) acids as well as *iso* and *anteiso* branched fatty acids with 15 carbon atoms were present in much lower concentrations. The *iso* and *anteiso* branched fatty acids are used as biomarkers for Gram-positive bacteria suggesting their involvement in biodegradation in some zones of the aquifer (Kaur et al., 2005). The fatty acid patterns of all depths were very similar although geochemical conditions were changing, suggesting that the fatty acid pattern did not respond sensitively to the geochemical conditions. However, the composition of fatty acids found in benzene amended *in situ* microcosms at marginal parts of the plume was more diverse suggesting a more diverse microbial community (Geyer et al., 2005). Further biomarker fatty acids specifically characterising some individual sulphate reducing bacterial strains were not observed (Kaur et al., 2005). In our studies we could not use the fatty acid pattern for a taxonomic interpretation.

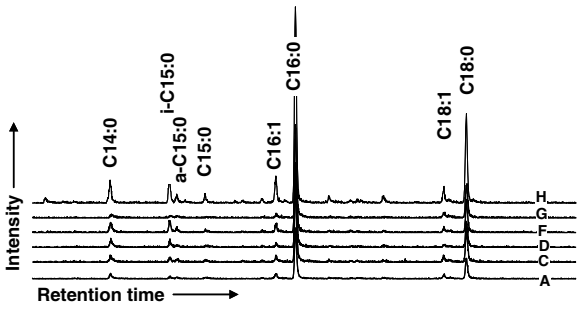

Fig. 4. The composition of the total fatty acid fraction (*m*/*z* 74) obtained from *in situ* microcosm experiment deployed at various depths (A–H) in well 23/00 at the field site Zeitz (Germany). The depths of the microcosm (A–H) and the position in the aquifer are shown in Fig. 2.

Isotope signature of fatty acids: The isotope compositions of fatty acids ($\delta^{13}C_{FA}$) extracted from the BACTRAPs amended with [$^{13}C_6$] benzene were between −28‰ and 439‰ (PDB) (Table 1). The variation in isotope composition may indicate the presence of organisms feeding on various carbon sources. Fatty acids with an isotope signature lower than −25‰ show the typical natural abundance of $\delta^{13}C_{FA}$ found in soil and aquifer materials (Pelz et al., 2001b; Pombo et al., 2002) and are typical for organisms using naturally occurring non-labelled carbon sources. In BACTRAP experiments with non-labelled substrate or without any substrate supply via Bio-Sep® beads all fatty acids showed natural abundances (−35 to −20‰, data not

Table 1
Carbon isotope composition of fatty acids extracted from *in situ* microcosms incubated with $^{13}C_6$ labelled benzene at monitoring well 23/00 (Zeitz, Germany) for 6 weeks

Depth	$\delta^{13}C_{FAME}$ [‰]							
	C14:0	*i*-C15:0	*a*-C15:0	C15:0	C16:1	C16:0	C18:1	C18:0
A	**63**	**89**	−22	−18	**330**	**62**	**22**	−21
C	−8	**90**	−19	−22	**439**	**47**	**61**	−15
D	−20	−17	−28	−23	**35**	−13	−21	−24
F	−17	**1**	−23	−23	**86**	**15**	−2	−25
G	−22	**134**	−23	−20	**174**	**20**	−6	−24
H	−22	−13	−22	−26	**24**	−3	−16	−26

A–H refers to incubation depth indicated in Fig. 2.

shown). Fatty acids with a positive carbon isotope signature (>0‰) clearly indicated that microorganisms incorporated the labelled carbon from the provided [$^{13}C_6$] benzene (Table 1). The enrichment of ^{13}C in fatty acids can only stem from the labelled substrate and thus clearly provided evidence of *in situ* benzene degradation.

BACTRAPs with ^{13}C-labelled benzene incubated in different geochemical zones showed variations in the ^{13}C-enrichment of fatty acids within the various depths although the composition of fatty acids did not change significantly. Hexadecenoic acid (C16:1) showed the highest incorporation of ^{13}C (439‰) next to *i*-C15 (134‰) and C16:0 (62‰). *a*-C15, C15:0, C14:0 and C18:0 displayed only low or even no incorporation of ^{13}C. The *i*-C15 generally showed a significantly higher labelling than *a*-C15 which was almost not labelled. The variation in $\delta^{13}C_{FA}$ clearly indicated that the organisms producing these FA grew on different carbon sources. Fatty acids displaying a higher incorporation of ^{13}C were very likely produced by organisms feeding on [$^{13}C_6$] benzene while fatty acids from bacteria with lower or no ^{13}C incorporation were likely not involved in the degradation of [$^{13}C_6$] benzene. Cross feeding by metabolites may channel labelled carbon into individual members of the microbial community.

In the upper zone of the aquifer the ^{13}C incorporation was higher, which may suggest a more intensive transformation of the [$^{13}C_6$] benzene. This might be a result of more attractive electron acceptors for degradation such as nitrate, manganese and iron as well as oxygen close to the water table. Nevertheless, also in the depth between 12 and 14 m indications for benzene degradation were found despite high benzene concentrations. Here sulphate reducing conditions were likely present.

On the other hand, labelling of biomass can be influenced by the availability of labelled benzene on the BACTRAPs relative to the unlabelled benzene from the groundwater itself. The concentration of benzene in the upper part of the plume was lower compared to deeper zones of the aquifer (Fig. 3). Therefore, benzene derived from the contamination might be available as a carbon source to a relatively higher amount at the depth of 12 and 14 m with benzene concentration up to 6 mmol l^{-1} (Fig. 3). The relative intensity of ^{13}C incorporation into biomass changed more with depth than the composition of the fatty acid fraction and might therefore reflect the relative intensity of benzene metabolism and geochemical conditions. Compared to previous investigations with [$^{13}C_6$] benzene amended *in situ* microcosms at marginal parts of the plume where fatty acids were labelled up to 13,000‰ (Geyer et al., 2005), the ^{13}C incorporation found in the multi level investigation was significantly lower (max. 439‰) suggesting a less intensive metabolism at the different depths of this monitoring well.

3.2. Gneisenau: upper Quaternary aquifer

The compositions of the total fatty acid fractions obtained from *in situ* microcosms loaded with [$^{13}C_6$] benzene and [$^{13}C_7$] toluene were compared in order to investigate the microbial communities growing on these different substrates. The *in situ* microcosms were exposed to the upper Quaternary aquifer Gneisenau, Germany (P10F) over 12 weeks and analysed for composition and isotope signatures of fatty acids. The patterns of fatty acids derived from toluene and benzene amended BACTRAPs incubated in the source zone of the Quaternary aquifer (P10F) were almost identical (Fig. 5). C18:0, C18:1 and C16:0 were dominating the fatty acid patterns. C18:1, *iso* and *anteiso* branched C15 compounds as well as C15:0 and C14:0 were present in minor concentrations. The similarity in fatty acid compositions

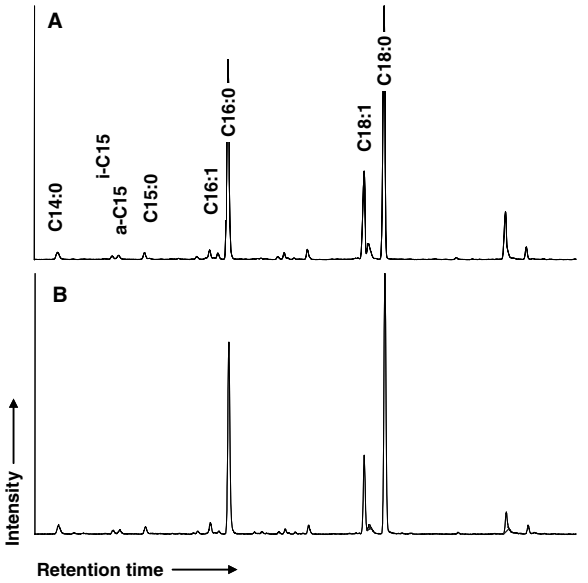

Fig. 5. Comparison of the composition of the total fatty acid fraction (*m*/*z* 74) obtained from *in situ* microcosms loaded with [$^{13}C_6$] benzene (A) and [$^{13}C_7$] toluene (B). The *in situ* microcosms were both exposed in the upper Quaternary aquifer (P10F) at the field site Gneisenau (Germany) for 12 weeks.

of both the benzene and toluene amended BACTRAPs (Fig. 5) indicated that the substrate did not affect the pattern of fatty acids suggesting that the lipid pattern is not a sensitive indicator to determine changes in the microbial community.

However, the isotope composition of the fatty acids from the benzene amended microcosm incubated in well P10F showed drastic differences in comparison to the toluene loaded one (Table 2). Only the hexadecanoic acid (C16:0) of the [$^{13}C_6$] benzene amended BACTRAP were found to be labelled. Unsaturated C18:1 and C18:2 isomers were only slightly or almost not labelled. The concentrations of other fatty acids were too low for the determination of their isotope composition (Fig. 5, Table 2). In contrast, the [$^{13}C_7$] toluene amended BACTRAP of well P10F showed a very strong incorporation of ^{13}C into fatty acids with carbon numbers between C14 and C18 of which hexadecenoic acid (C16:1) was most intensively labelled (45,000‰). Interestingly the fatty acids with 18 carbon atoms were labelled two orders of magnitude lower. The significant high labelling of fatty acids from the toluene amended BACTRAP demonstrated extensive transformation of toluene by some community members whereas benzene carbon was only assimilated to a limited extent. Obviously, other members of the community, producing the significantly lower labelled fatty acids, used other carbon sources in addition to the labelled contaminants.

3.3. Gneisenau: Lower Cretaceous aquifer

In situ microcosms were also applied to evaluate the biodegradation of benzene and toluene in the deeper fractured Cretaceous aquifer. This aquifer was a hundred times more contaminated with benzene (up to 45 mg l^{-1}) than the upper Quaternary aquifer. BACTRAPs loaded with [$^{13}C_6$] benzene, [$^{13}C_7$] toluene and with non-labelled substrates were exposed up stream from the source zone (P8T) and in the centre of the plume (P11T). A higher enrichment of ^{13}C in fatty acids, e.g. in C16:1 (96,000‰) and C16:0 (9700‰), was only observed in the toluene amended microcosms up stream from the source

Table 2
Carbon isotope composition of fatty acids extracted from *in situ* microcosms incubated with $^{13}C_6$ labelled benzene and $^{13}C_7$ labelled toluene in the upper Quaternary monitoring well (P10F) and in the lower Cretaceous monitoring wells (P8T, P11T) at the field site Gneisenau (Germany) for 12 weeks

	$\delta^{13}C_{FAME}$ [‰]				
Well:	P8T	P10F		P11T	
Fatty acid	^{13}C-Toluene	^{13}C-benzene	^{13}C-toluene	^{13}C-benzene	^{13}C-toluene
14:0	n.d.	n.d.	2800	n.d.	390
i-15:0	41,000	n.d.	14,000	n.d.	30
a-15:0	n.d.	n.d.	1800	n.d.	−21
15:0	n.d.	n.d.	580	172	n.d.
i-16:0	n.d.	n.d.	7600	n.d.	−42
16:1	96,000	n.d.	46,000	n.d.	140
16:0	9700	9	3600	−9	−18
18:2-18:1-Cluster	400	−17	540	−16	−19
18:0	3	−27	290	−30	−26

n.d., not determined due to low concentration.

area (P8T) in relatively uncontaminated zones of the deeper fractured aquifer (Table 2). The fatty acids of benzene or toluene amended BACTRAPs from the source zone (P11T) in the deeper aquifer were only slightly labelled in both cases (Table 2). The fatty acids occurring in relatively higher concentrations such as C16:0, unsaturated C18 isomers and C18:0 were almost not labelled in comparison to fatty acids commonly present in lower concentrations such as C14-, C15-species and C16:1 (Table 2). This suggests that only a minor part of the community was involved in the contaminant degradation.

The results show that the transformation of contaminant derived carbon into fatty acids was higher in toluene compared to benzene amended BACTRAPs, which is in accordance to earlier findings that toluene is more easily degradable under anaerobic conditions than benzene (Wiedemeier et al., 1999; Cunningham et al., 2001; Da Silva and Alvarez, 2004). In a direct comparison of parallel experiments with labelled toluene and benzene, the amount of ^{13}C transferred into fatty acids may be used as a direct indicator for the *in situ* degradability of the substrates. The highest enrichment of ^{13}C into fatty acids was observed in BACTRAPs loaded with labelled toluene indicating a higher degradation capacity for toluene compared to benzene. As the *in situ* microcosms were exposed in an almost uncontaminated zone of the deeper aquifer a significant potential to degrade the contaminants was present even in almost non-contaminated zones.

To sum up the results, Bio-Sep® beads spiked with ^{13}C BTEX can be used to investigate the *in situ* biodegradation of BTEX compounds semi-quantitatively by the amount of incorporated ^{13}C in lipid derived fatty acids. The intensity of ^{13}C incorporation into biomass (FA) provides valuable information on microbial diversity and on the relative amount of metabolism. The quantification of biodegradation was not possible in these experiments, but with an absolute quantification of fatty acids and an estimation of the proportion of fatty acids in anaerobic bacteria it may be possible to quantify the incorporation into the biomass. Thus further development is needed to apply the BACTRAP system to quantify *in situ* biodegradation.

3.4. Molecular biological analysis of microbial communities on BACTRAPs

In order to characterise the contaminant degrading microbial community in the aquifer the biodiversity of the microbial biomass can be analysed by cultivation independent molecular biological approaches based on PCR-amplified partial 16S rRNA genes from total DNA extracted from groundwater samples (Scow and Hicks, 2005). In the present study, total DNA was extracted from the BACTRAPs and the PCR products amplified from the different samples (wells) were compared to each other by their SSCP profiles (Fig. 6). This technique is now widely used in microbial ecology to compare the structural diversity of microbial communities (Dohrmann and Tebbe, 2004). In addition to comparing the structural diversity of the bacterial community from different samples, bands of the SSCP-profiles can be DNA-sequenced in order to characterise individual members of the bacterial community (Dohrmann and Tebbe, 2004).

Most of the SSCP profiles generated in this study were relatively similar to each other, indicating a reproducible colonisation pattern of the bead material independently of the geochemical conditions in the investigated groundwater wells (Fig. 6). For most samples analysed, the bacterial community compositions on the benzene and toluene loaded microcosms were only slightly altered in comparison

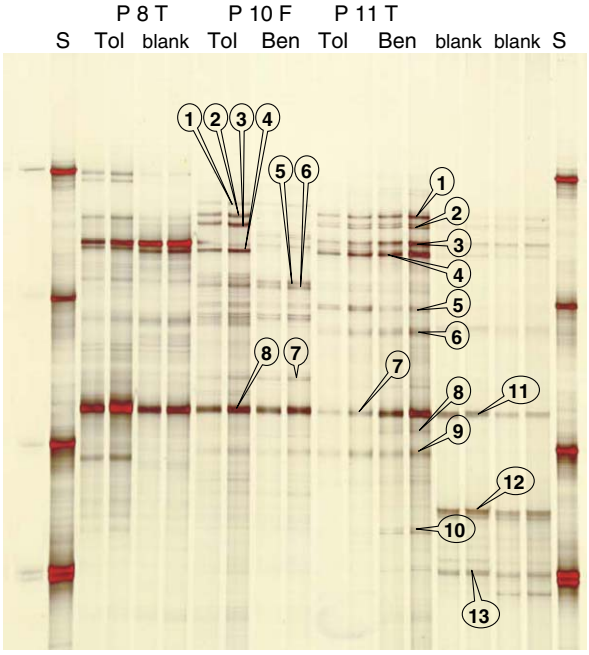

Fig. 6. SSCP gel of PCR amplified 16S rDNA isolated from BACTRAPs loaded with $^{13}C_7$ toluene or $^{13}C_6$ benzene after exposure (84 d) in a contaminated aquifer. The bands (numbers) are sequenced for identification (see Table 3).

Table 3
Characterisation of rRNA genes isolated from BACTRAPs incubated in the lower (P11T) and upper (P10F) aquifer at the field site Gneisenau (Germany)

Band	Clone	Length [bp]	Identity [%]	Closest relative in database (GenBank Accession No.)	Phylogenetic group	Source of sequence or isolate
Upper aquifer (P10F)						
1	P10F-01c	371	99.7	Uncultured bacterium (AF534262)	Deltaproteobacteria	Marine and freshwater sediments
1	P10F-01a	368	99.5	Uncultured bacterium (DQ404728)	Candidate division OP3	Contaminated sediment
2	P10F-02b	371	98.4	Uncultured bacterium (AF050590)	Firmicutes	Hydrocarbon- and chlorinated-solvent-contaminated aquifer
2	P10F-02b	374	99.5	Uncultured bacterium (AY050586)	Deltaproteobacteria	Monochlorobenzene contaminated groundwater
3	P10F-03c	371	97.6	unidentified bacterium (AF058010)	Deltaproteobacteria	PCB-dechlorinating enrichment culture
4	P10F-04c	369	98.9	Uncultured bacterium (AY475201)	Alphaproteobacteria	Metal-rich and acidic river water
6	P10F-06b	369	100	*Ralstonia pickettii* (AY741342)	Betaproteobacteria	Clinical origin
7	P10F-07a	367	99.7	Uncultured bacterium (AJ306737)	Bacteroidetes	1,2-Dichlorpropane dechlorinating consortium
8	P10F-08b	369	98.9	Uncultured bacterium (AB205680)	Alphaproteobacteria	Denitrifying activated sludge
Lower aquifer (P11T)						
1	P11T-01a	368	97.3	Uncultured bacterium (DQ404728)	Candidate division OP3	Contaminated sediment
1	P11T-01b	371	99.4	*Geobacter pelophilus* (U96918)	Deltaproteobacteria	Dissimilatory Fe(III)-reducing bacteria
2	P11T-02a	371	98.7	Uncultured bacterium (AY945913)	Betaproteobacteria	Quinoline-removal bioreactor
3	P11T-03a	371	99.2	Uncultured bacterium (AF104274)	Deltaproteobacteria	Benzene-contaminated aquifer sediments
5	P11T-05b	369	99.2	Uncultured bacterium (AB240520)	Betaproteobacteria	Rhizosphere biofilm of *Phragmites* at river
5	P11T-05a	369	100	*Ralstonia pickettii* (AY741342)	Betaproteobacteria	Clinical origin
6	P11T-06d	371	98.4	Uncultured bacterium (AY250093)	Betaproteobacteria	Napthalene-contaminated sediment
6	P11T-06c	367	97.3	Uncultured bacterium (AB240238)	Bacteroidetes	Reed bed reactor
7	P11T-07c	369	99.2	Uncultured bacterium (AB205680)	Alphaproteobacteria	Denitrifying activated sludge
9	P11T-16b	366	92.3	Uncultured bacterium (AB205753)	Firmicutes	Denitrifying activated sludge
10	P11T-10b	367	99.4	Uncultured bacterium (AJ519404)	Bacteroidetes	Uranium mining waste pile
13	P11T-14d	371	88.9	Uncultured bacterium (UEU81707)	Chlamydiae	Anaerobic digester

to the non-amended ones (blank). However, the fact that highly labelled microbial fatty acids were extracted from the ^{13}C experiments indicated a substrate degradation and incorporation into biomass to a different extent (Table 2). This may show a coordinated degradation by certain members of the microbial community colonising the *in situ* microcosms and a channelling of the ^{13}C carbon into the microbial food web. Even though some fatty acids were highly labelled, the isotope composition showed that these organisms also used other carbon sources. In the highly contaminated part of the aquifer, benzene and toluene were presumably the major carbon sources but other BTEX compounds and minor contaminants as well as humic substances were certainly present and may be used as carbon sources as well. In addition, some fatty acids in ^{13}C experiments were not labelled and indicated that microbes not involved in pollutant degradation were also colonising the bead material.

The comparison of the non-amended (blank) with the loaded BACTRAPs showed that the SSCP- and FA patterns were only slightly different (Figs. 5 and 6). This reflects the fact that the bead material (activated carbon) of the blanks extracted a certain amount of the contaminant, i.e. benzene, from the contaminated aquifer leading to similar conditions in the non-loaded microcosms compared to the benzene loaded ones. The SSCP- and FA patterns from the toluene loaded BACTRAPs were also only

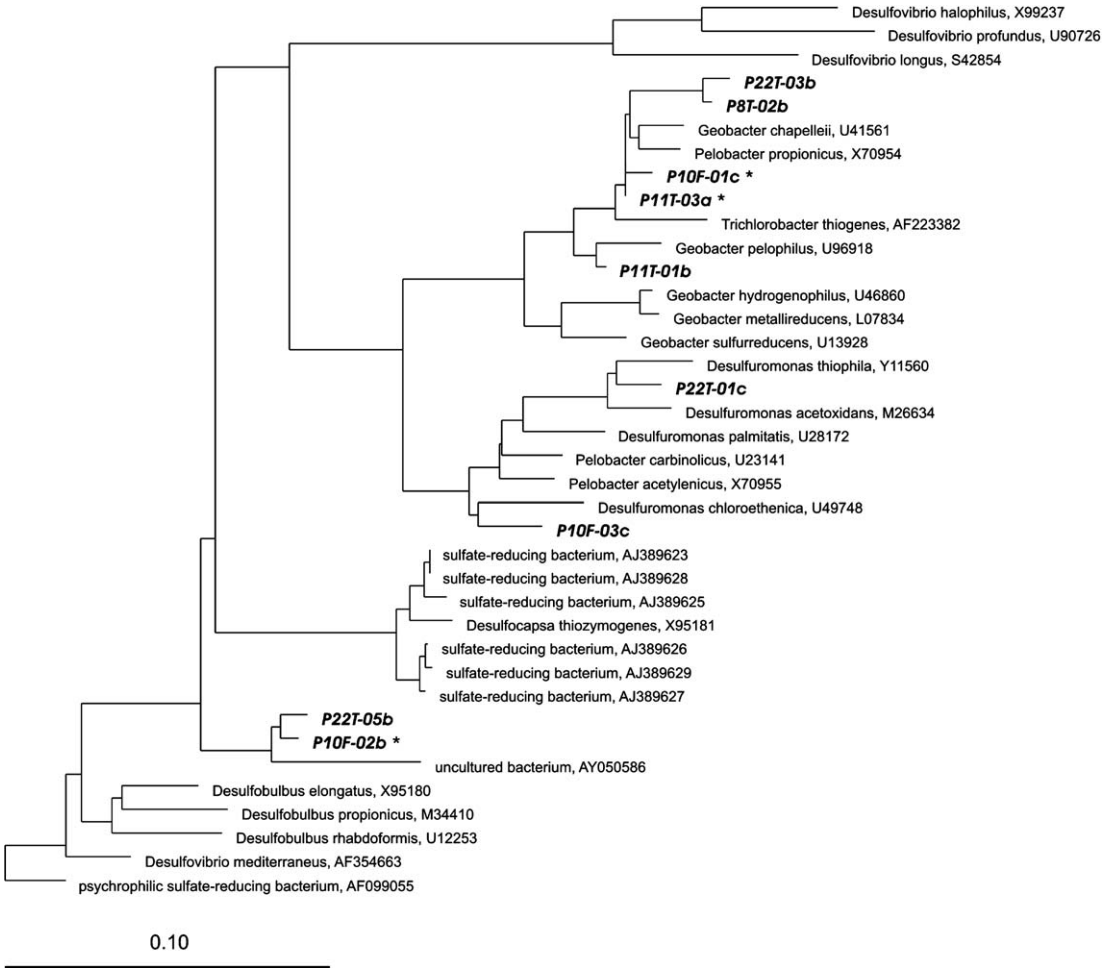

Fig. 7. Phylogenetic analysis (maximum likelihood method) of PCR-amplified partial 16S rRNA genes from the group Deltaproteobacteria, retrieved from BACTRAPs exposed at the field site Gneisenau. Clones are indicated by their source of isolation (P10F for upper and P11T for lower aquifer) followed by their clone number. *, indicates sequences which were found at two subsequent sampling dates in the same well.

slightly different from the non-amended (blank) and benzene BACTRAPs showing that toluene degraders were members of the colonising community.

Digital image analyses showed that the profiles of the different wells clustered together mostly independent from the loaded substrates (data not shown). This indicated a dominant effect of the environmental conditions of each well on the communities developing on the BACTRAPs and a minor influence of the supplied substrate. Therefore, no signals with respect to new emerging bands showed the colonisation of specific contaminant degraders on the loaded *in situ* microcosms. The intense bands of the patterns from the low contaminated area (P8T) indicated a good growth on the microcosms whereas the generally smaller bands from the wells of the contaminated area (P10F and P11T) showed lower biomass accumulation and may point to a certain toxicity of the benzene in that area (Fig. 6). The identified DNA-sequences of the SSCP-profiles were mostly related to sequences from yet uncultivated bacterial species (Table 3 and Fig. 7). Phylogenetic analyses revealed a community dominated by iron and sulphate reducing bacteria particularly of the *Geobacter* and *Desulfuromonas* groups (Lovley and Phillips, 1988; Zhang et al., 2003). These groups of organisms are known to be involved in contaminant degradation in anaerobic aquifers. This shows that iron and sulphate reducing microbial communities are likely to be involved in the contaminant degradation and that iron and sulphate were used as terminal electron acceptors at this field site.

The molecular biological data supported the results of the hydrological and geochemical analyses and provided valuable information about the biodiversity of the degrading microbial community. In addition, the BACTRAP approach with labelled contaminants provided an excellent tool for assessment and proving the microbial *in situ* activity in contaminated aquifers. However, the key organisms related to contaminant degradation could not be identified. In future, growth experiments to investigate the kinetics of the colonisation traced by quantitative PCR may elucidate the development of communities and may provide indications for organisms supported by the test substrate. Provided that the total biomass on BACTRAPs is sufficient, stable isotope probing (SIP) may also be applicable to identify organisms which used the ^{13}C labelled contaminants primarily as a carbon substrate (Lueders et al., 2004; Manefield et al., 2004).

3.5. Investigation of metabolic pathways

The extracted carboxylic acid fraction from *in situ* microcosm experiments with [^{13}C]-α-toluene contained ^{13}C labelled metabolites which could be easily identified by GC–C–IRMS analysis, due to the very strong signal caused by the ^{13}C labelled carbon although their absolute concentration was very low (Fig. 8). An examination of the labelled peak by GC–MS gave a spectrum indicative for benzylsuccinic acid dimethyl ester (Fig. 9). The non-labelled analogue was found in the carboxylic acid fraction of the BACTRAPs amended with non-labelled toluene. The presence of benzylsuccinate was further confirmed by comparison of the mass spectra as well as the retention time of the derivatized authentic standard. The formation of benzylsuccinate is indicative for the anaerobic toluene degradation pathway which is employed by sulphate and iron reducing microorganisms able to degrade toluene (Heider et al., 1999; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Benzylsuccinate has been used as an indicator for anaerobic toluene degradation in contaminated aquifers before (Beller, 2000; Griebler et al., 2004). Therefore, the benzylsuccinic acid extracted from the *in situ* microcosm is a good indicator for the biogeochemical conditions governing the toluene degradation in this particular aquifer.

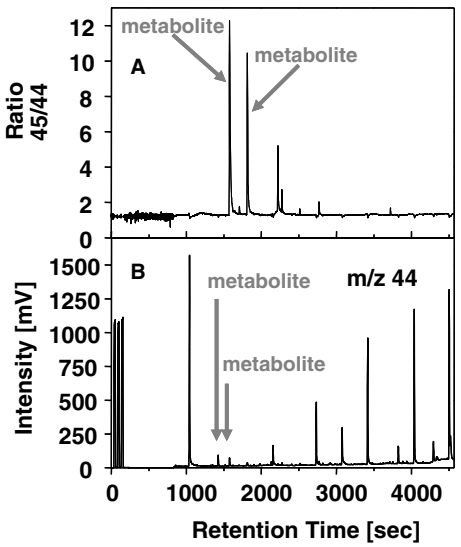

Fig. 8. GC–C–IRMS-chromatogram of the carboxylic acid fraction extracted from a [^{13}C]-α-toluene amended BACTRAP incubated at the field site Zeitz (Germany). The metabolites gave very strong signals in the 45/44 ratio plot (A) although their absolute concentration displayed by the amount of CO_2 (m/z 44) was relatively low (B).

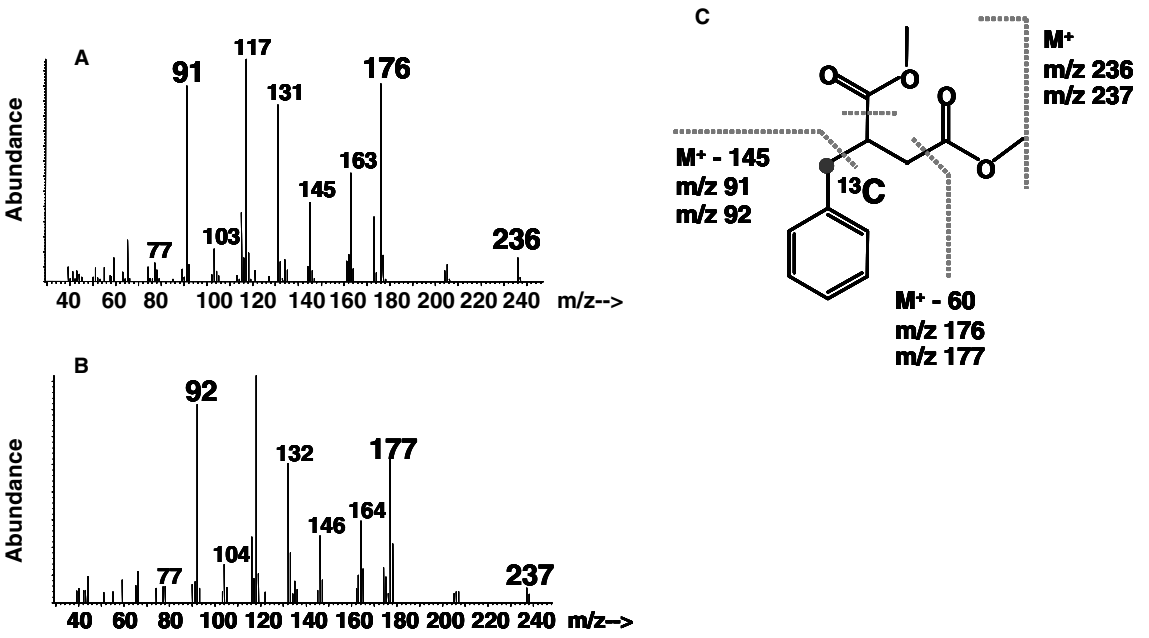

Fig. 9. Mass spectra of benzylsuccinate extracted from *in situ* microcosms incubated 92 d within an anaerobic aquifer at the field site Zeitz (Germany). (A) BACTRAP loaded with non labelled toluene, (B) BACTRAP loaded with [^{13}C]-α-toluene. The fragmentation pattern of ^{13}C labelled benzylsuccinate shows that the ^{13}C atom was found at the position of the former methyl group (C).

This clearly shows that BACTRAPs can also be useful to identify degradation pathways in the aquifer by analysing the potential metabolites. Reusser and Field (2002) applied push and pull experiments with deuterium labelled toluene to verify *in situ* toluene degradation in a contaminated aquifer. Although deuterium labelled contaminants may be useful tracer compounds to provide evidence for *in situ* degradation, much higher amounts of labelled substances are needed during push and pull experiments in comparison to the BACTRAP approach. As described here, the application of *in situ* microcosms can significantly reduce the amount of labelled material and gives in principle similar information as obtained in push and pull experiments. When using ^{13}C labelled compounds, the transformation into biomass can be traced by analysing the isotope composition of fatty acids and perhaps may also provide kinetic information in future.

4. Conclusions

In situ microcosms with ^{13}C labelled substrate can be used to demonstrate *in situ* biodegradation of difficult degradable contaminants such as benzene and toluene under anoxic conditions by analysing the isotope composition of fatty acids extracted from the biomass grown on the BACTRAP. The isotope composition in combination with the pattern of fatty acids indicated that a complex microbial community was colonising the BACTRAPs, but apparently not all community members were involved in the contaminant degradation. The fatty acid patterns were not of taxonomic value and no sensitive indicator to determine changes in the microbial community within various environmental conditions but isotope composition was very useful to provide evidence for *in situ* degradation. Molecular biological analyses showed that mainly uncultured iron and sulphate reducing communities were colonising the BACTRAPs which reflected the biogeochemical conditions and terminal electron accepting processes in the aquifer. By analysing the mass spectra of metabolites we are able to identify the *in situ* degradation pathways. Benzylsuccinate found on toluene amended BACTRAPs indicated that toluene was degraded via the benzylsuccinate pathway which is typical for sulfidogenic and iron reducing conditions.

In summary, BACTRAPs give qualitative information on *in situ* biodegradation of contaminants.

Furthermore, with additional molecular biological methods we were able to identify the structure of the microbial community growing on the BACTRAPs which may reflect the *in situ* microbial community to some extent. Compared to laboratory microcosm studies, the advantage of the BACTRAPs is that the incubation takes place under environmental conditions directly in the aquifer at the field site. At present, mass balances were not possible in BACTRAP experiments which is certainly a limitation compared to closed laboratory systems.

In terms of the assessment of *in situ* biodegradation in contaminated aquifers *in situ* microcosms with labelled substances are relatively simple, low-cost assays to gain information on the potential of the microbial community able to degrade contaminants within a reasonable timeframe under *in situ* conditions.

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Anhang D

Assessment of Microbial *In Situ* Activity in Contaminated Aquifers (2006)

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Assessment of Microbial In Situ Activity in Contaminated Aquifers

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Microbial ecologists and environmental engineers share the interest in identifying the key microorganisms responsible for compound turnover in the environment and in estimating the respective transformation rates. For the successful application of Natural Attenuation processes, a reliable assessment of the in situ turnover of a contaminant in an aquifer is essential. Here, we review and present new details of two recently developed approaches concerning the assessment of in situ biodegradation: *(i)* determination of biodegradation caused by microbial metabolism in a contamination plume by stable isotope fractionation analysis (SIFA) and *(ii)* determination of the actual degradation under the respective environmental conditions in the aquifer by using in situ microcosms (BACTRAPS®) amended with ^{13}C-labeled substrates as tracer compounds. Based on stable isotope fractionation analysis, the degradation occurring under anoxic biogeochemical conditions at a respective site can be calculated for the entire plume. This has been shown for benzene and toluene at the Zeitz site and partly for chlorobenzene at the Bitterfeld site. By use of the in situ microcosm approach with ^{13}C-labeled compounds, the microbial in situ degradation under strictly anaerobic conditions could be proven for benzene and toluene in Zeitz and for chlorobenzene in Bitterfeld. The transformation of ^{13}C-carbon of the labeled substrate into microbial fatty acids confirmed the assimilation of the pollutant resulting in the formation of biomass. In addition, metabolites such as benzylsuccinic acid were found in the toluene-amended microcosms indicating anaerobic degradation of toluene. This result corresponds to the geochemical conditions found at the field site and therefore, the microcosm approach with ^{13}C-labeled compounds can be used to assign the predominant in situ degradation pathways in a contaminated aquifer. Since fatty acids profiles alone are often too unspecific for a community analysis at species level, the composition of the microbial communities was analyzed by genetic profiling and sequencing of partial 16S rRNA genes PCR-amplified from total DNA extracted directly from the microcosms. Sequences retrieved from the microcosms indicated a dominance of not yet cultivated bacteria. Several sequences were phylogenetically closely related to sequences of bacteria known to be iron and sulfate reducers, typically found at sites polluted with BTEX and/or mineral oil. The results show that the current methods for monitoring microbial in situ activity at present stage are valuable tools for improving environmental control of compound turnover and will speed up engineering approaches.

1 Introduction

Since Winogradski's column experiments, microbiologists and biochemists have long tried to link the transformation of compounds in the environment with the key microorganisms involved in the respective transformation. This linking is a crucial prerequisite for shedding light into the black box of real world microbial processes. From a technical perspective, it is also necessary to have a detailed knowledge about the key processes for purposes of control and successful engineering. In the past decade, methodological advances in linking the structure of microbial communities with the actual activity and function of their members generated dramatic progress in the knowledge of microbial mediated environmental processes and led to the development of new innovative approaches such as waste water treatment using the Anammox process [68].

The fate of pollutants in contaminated aquifers is mainly governed by microbial degradation. Abiotic processes such as dispersion, dilution, sorption, and volatilization may contribute to a decrease in concentration but do not lead to a significant reduction of the mass of contaminants. Therefore, the evaluation of in situ biodegradation is essential for the implementation of Natural Attenuation (NA) concepts in groundwater management strategies (Oswer directive; [80]). Plate counting methods were used in the past for the assessment of the number of microbes present, however, these methods only provide information about the culturable species. Advanced conventional methods apply the laborious quantification of electron balances of typically complex electron donor-terminal electron acceptor interactions. Calculations then relate the decreasing contaminant concentrations to the consumption of electron acceptors in order to assess biodegradation processes [14]. When tracing the fate of single compounds in contaminant mixtures, this approach is complicated particularly on the field scale in the very heterogeneous natural environment where several electron acceptor-donor interactions compete. Laboratory microcosm

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studies are often used to obtain information on in situ biodegradation. However, this approach is not reliable because the majority of microorganisms have not yet been cultivated [64, 84]. In particular, the reproducible cultivation of anaerobic bacteria that degrade typical contaminants such as BTEX and PAHs can be difficult and may lead to misinterpretation of the intrinsic biodegradation potentials. Laboratory microcosm studies or percolation column experiments, simulating the contaminant degradation under controlled water flow conditions, are time-consuming; furthermore, the conditions in the laboratory are mostly different to the natural environmental conditions resulting in certain limitations in the reliability of the data acquired.

Recently, the ^{13}C-enrichment of phospholipid-derived fatty acids (PLFA) related to the consumption of ^{13}C-labeled substrates was used to characterize bacterial toluene degradation in soil, sediment or aquifer microcosms [25, 54, 55] and to trace the assimilation of toluene along a food chain [43], [58]. The ^{13}C-tracer concept can provide useful information on carbon fluxes, metabolites and microbial food webs. At the field scale, microbial incorporation of ^{13}C-labeled acetate into biomarker molecules such as PLFA and DNA was successfully assessed to indicate microbes which were suggested to be responsible for the reduction of uranium(VI) [12]. A mesocosm was designed to monitor the in situ dynamics of the microbial community in a BTEX polluted aquifer [29]. In this study, uncontaminated aquifer material was incubated either in the uncontaminated area or in a contaminated area of the aquifer located nearby, and the developed bacterial communities were analyzed by molecular biology techniques. Other authors applied the electron donors glucose and ethanol to acidic uranium and nitrate contaminated groundwater. Fortunately, they were able to show the increase of 16S rRNA gene sequences related to previously cultured metal reducing δ-proteobacteria by 1–2 orders of magnitude using quantitative PCR techniques and time resolved sampling at the site [52]. These approaches provide valuable information on the functional biodiversity in soils and sediments. However, from a perspective of the contaminant history they do not provide reliable information on the compound turnover actually occurring under in situ conditions in an aquifer. In addition, it is difficult to provide any predictive data on the future fate of the residual contaminants under varying environmental conditions. These approaches may even fail, if organisms with necessary physiological properties were not cultured previously, or if the physiological capacities of the respective organisms are not generally expressed even though the organisms are present in the contaminated aquifer. Such effects are known from *Dehalococcoides* sp. [31, 35].

The objective of the present work is to review the recently developed approaches focusing on the assessment of the in situ biodegradation in contaminated aquifers. Two approaches will be described dealing with *(i)* determining the cumulated biodegradation in a contamination plume by use of fractionation of stable isotopes at natural abundance during microbial metabolism and *(ii)* the assessment of the actual degradation under the respective environmental conditions in the aquifer. The latter approach employs in situ microcosms (BACTRAPs®) amended with ^{13}C-labeled substrates such as toluene, benzene, and monochlorobenzene and enables the reliable quantification of the actual degradation. It will also enable the identification of the microbial key players in the near future. We present new results from applying the two approaches at field sites.

2 Materials and Methods

2.1 Chemicals

The chemicals and solvents were obtained in p.A. quality from Merck unless otherwise stated. [$^{13}C_6$] benzene, [^{13}C]-α-toluene and benzylsuccinic acid were obtained from Sigma-Aldrich (St. Louis, USA). [$^{13}C_6$]-monochlorobenzene and [$^{13}C_7$]-toluene were purchased from Chemotrade Leipzig (Germany). All the isotopically labeled compounds had a chemical purity higher than 99 %.

2.2 Field Sites (Zeitz, Bitterfeld)

Zeitz (Saxony-Anhalt, Germany)

The benzene, toluene, ethylbenzene and *o,m,p*-xylene (BTEX) contaminated aquifer is located in the area of a former hydrogenation plant close to the city of Zeitz in Saxony-Anhalt, Germany. A detailed description of the site hydrogeology and hydrochemical conditions of the contamination plume at the SAFIRA (Remediation research in regional contaminated aquifers) site was given in previous studies [18, 86]. The thickness of the upper aquifer varies between 4 to 6 m. Depth discrete investigation of geochemical and isotope parameters in Zeitz have been previously described [76]. In the source area BTEX exceeded concentrations of 900 mg/L and benzene and toluene were present in concentrations up to 850 mg/L and 50 mg/L, respectively. Ethylbenzene and xylenes are typically present in concentrations lower than 3 mg/L. The groundwater was sampled from the wells indicated in Figs. 2A and B. At the test site, the predominant electron acceptor used for biodegradation was sulfate [86] and the contribution of methanogenic processes in the microbial transformation in the BTEX-plume is of minor importance [18]. Other electron acceptors such as oxygen, nitrate and iron play a minor role for the overall biodegradation processes at the site.

Bitterfeld (Saxony-Anhalt, Germany)

The monochlorobenzene (MCB) contaminated aquifer is located near the city of Bitterfeld in Saxony-Anhalt, Ger-

many. Concentrations up to 30 mg/L of MCB and lower concentrations of chlorinated aliphatic compounds (<5 mg/L), benzene (<0.77 mg/L), and dichlorobenzenes (<3 mg/L) were found in the aquifer [89]. Due to the 150-year history of the chemical industry at the site, the time of spillage is not exactly known but chlorobenzenes have been produced at the industrial site since the first half of the last century. The hydrogeological situation is characterized by a shallow Quaternary aquifer system consisting of glaciofluvial sand and gravel and a deeper Tertiary aquifer system of micaceous sand overlying glauconite sand. A lignite layer overlaid by a Miocene clay layer formerly separated the Quaternary and Tertiary aquifer systems, which has been partially removed by open-cast mining. Today the two aquifer systems are hydraulically connected at several locations. A Rupelian clay layer is the major regional aquitard of the Tertiary aquifer system [27, 28].

The MCB plume stretches down-gradient at the bottom of a Quaternary hydrogeological channel structure in a southeasterly direction [34]. This Quaternary channel is located above the lignite layer, the local aquitard, separating the Quaternary from the underlying Tertiary aquifer. Oxygen concentrations in the aquifer were generally below 1 mg/L. Significant nitrate concentrations were only found in the upper layers of the aquifer indicating that nitrate was not a relevant terminal electron acceptor (TEA). Some iron and manganese reduction was indicated by low Fe(II) and Mn(II) concentrations. Elevated sulfide concentrations associated with sulfate depletion indicated sulfate reduction, and sulfate concentrations of up to 1.48 g/L suggested that sulfate was the principle TEA in this aquifer [34]. Methane was not found in the aquifer indicating that methanogenesis was a very unlikely electron acceptor process. The groundwater in Bitterfeld was sampled in a cross section A-B between 17 and 21 m below ground (Left fringe: wells SAF11, SAF12, SAF27, SAF28, and right fringe: SAF7, SAF30, SAF31, SAF23, see Figs. 7 and 8) of the lower aquifer in monitoring wells which are perpendicular to the contamination plume as described in details elsewhere [34]. In addition, in well SAF 4/97 the upper, Quaternary aquifer was investigated during a depth discrete multilevel sampling campaign.

2.3 Isotope Fractionation and Calculations

The carbon isotope composition of contaminants and fatty acids is reported in δ-notation (‰) relative to the Vienna-PeeDee-Belemnite-Standard (V-PDB) with $^{13}C/^{12}C = (11237.2 \pm 2.9) \times 10^{-6}$ [15] (Eq. (1)):

$$\delta^{13}C[‰] = \left(\frac{(^{13}C/^{12}C)_{Probe}}{(^{13}C/^{12}C)_{Standard}} - 1 \right) \times 1000 \quad (1)$$

The determination of the fractionation of stable isotopes caused by primary enzymatic transformation reaction of a compound during microbial degradation can be described with the Rayleigh equation [30, 41]. The Rayleigh equation (Eq. (2)) can be used to relate the change in concentration (C_t/C_0) to the instantaneous change in isotope composition (R_t/R_0) by the isotope fractionation factor (α):

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0} \right)^{\left(\frac{1}{\alpha} - 1\right)} \quad (2)$$

R_0 and R_t are the isotope ratios at the beginning of the transformation reaction (t = 0) and after a given time (t), respectively. C_0 and C_t are the corresponding concentrations at the beginning of the reaction (t = 0) and after a distinct time (t), respectively. The isotope fractionation factor α quantifies the extent of isotope fractionation. The isotope fractionation is often reported as the enrichment factor ε [‰] = (α – 1) × 1000.

Isotope fractionation factors are determined in laboratory reference experiments with microcosms, column experiments or pure cultures where microbial degradation is the only sink of contaminants. In this case the change in isotope ratio (R_t/R_0) can be related unequivocally to the change in concentration (C_t/C_0). In a double logarithmic plot of C_t/C_0 and R_t/R_0, the isotope fractionation factor can be determined from the slope of the linear regression curve. Isotope fractionation factors (αD, αC, αCl) published until 2004 can be obtained from recent reviews [44, 69] or from a frequently updated data base (www.isodetect.de).

The isotope fractionation concept for quantifying the microbial in situ degradation is based on the fact that in situ biodegradation is the major process altering the isotope composition of priority contaminants such as BTEX, chlorobenzenes and chlorinated ethenes in groundwater to a significant extent. Other processes such as sorption and volatilization may affect the isotope composition but do not alter the isotope composition to a significant extent in comparison to biodegradation. Therefore, the isotope composition of contaminants in groundwater provides information about the cumulated extent of intrinsic transformation. The isotope fractionation caused by biodegradation leads to an enrichment of heavy isotopomeres in the residual fraction of the contaminant. A modified Rayleigh equation is used to quantify the microbial in situ degradation degree (Biodegradation; B [%]). B [%] represents the concentration decrease expected along a theoretical streamline plug flow without mixing and a single degradation process with a constant isotope fractionation factor. The change in concentration (C_t/C_0) down gradient from the source of contaminants is calculated employing the changes in the isotope ratio between the source (R_o) and a monitoring well (R_t) and an appropriate isotope fractionation factor (α) (Eq. (3)).

$$B[\%] = \left(1 - \frac{C_t}{C_0}\right) \times 100 = \left[1 - \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\frac{1}{\alpha} - 1}\right)}\right] \times 100 \quad (3)$$

2.4 Preparation and Incubation of In Situ Microcosms

The in situ microcosm experiments were performed using the BACTRAP system described elsewhere [10, 20]. Briefly, Bio-Sep® beads (provided by K. Sublette, University of Tulsa, Tulsa, USA) were loaded with contaminants as a carbon source for microorganisms. The spherical beads, 2 to 3 mm in diameter, consist of powdered activated carbon (PAC) incorporated within an aramid polymer matrix (Nomex®). The beads have a porosity of 75 %, an internal surface area greater than 600 m^2/g and outer pores of 1–10 microns [53, 90]. The beads were heated at 300 °C for 4 hours to remove organic residues and then 0.5 g of beads per trap were transferred into perforated Teflon® tubes and fixed with glass wool plugs at both ends. The filled microcosms were autoclaved at 121 °C for sterilization and hydration of the bead material and then air-dried for 24 hours. The microcosms were loaded with the substrates (benzene, toluene or chlorobenzene) using gas phase under reduced pressure. The substrate and the microcosms were placed in small glass containers and evacuated for at least 48 hours to 60 mbar in order to allow uniform adsorption of the contaminants on the beads. In order to keep the beads anoxic, the vacuum was released by filling oxygen free water into the glass container and the beads were stored under anoxic conditions until their deployment in the monitoring wells.

The BACTRAPs for the microcosm experiments in Zeitz were loaded with [$^{13}C_6$]-benzene to a concentration of about 88 mg/g bead material and with [^{13}C]-α-toluene to 112 mg/g. The microcosms were deployed in the well Saf Zz 32/02 at a depth of 14–15 m (4 m below the groundwater table). The in situ microcosms for the experiments in Bitterfeld were amended with [$^{13}C_6$]-chlorobenzene to a concentration of 38 mg/g. For control experiments, the BACTRAPs were loaded with non-labeled benzene, toluene or chlorobenzene in similar concentration. Material from these control microcosms was used for molecular biological studies and to investigate the isotope composition of fatty acids at natural abundance.

A multi level packer system (MLPS) was used for depth discrete sampling from a groundwater well in Bitterfeld [34, 66], which was equipped with a filter screen from 4.5 to 22.3 m depth below ground. The multi-level packer system is a water-filled sock tube inserted into the well, which allows various sampling systems to be separated from each other at different depths. Small submersible pumps allowed depth-specific sampling without cross-currents. A detailed description of the multi-level sampling system is given by [66]. The microcosms were deployed in the well for 49 d at a depth of 10.7 m below the surface. The well is located in the anoxic central part of the plume and by the conventional sampling 0.3 mg MCB/L was found. In the depth discrete sampling campaign, concentrations below 1.0 mg/L were found up to the depth of 18 m and the concentration increase to more than 13 mg/L in the deeper part of the well.

2.5 Groundwater Sampling and Analyses

Groundwater sampling for the determination of concentrations and isotope signatures was performed according to good sampling practice. No specific efforts are needed and the samples can be taken in parallel during conventional groundwater monitoring campaigns. Sampling techniques are described in the literature for example according to the German Industrial Norm [1, 2]. The biological activity in the samples can be stabilized by adding $HgCl_2$, sodium azide, or other stabilizers depending on the target compound. Water samples with target compounds resistant to hydrolysis such as hydrocarbons can be stabilized by rising or lowering the pH. For the sampling of BTEX and monochlorobenzene (MCB), a 1 L bottle was completely filled with groundwater. NaOH pellets were added in order to raise the pH to at least 10 and the bottle was closed with a Teflon lined screw cap.

The sensitivity of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) systems is lower than conventional gas chromatography-mass spectrometry (GC-MS) techniques and therefore GC-C-IRMS requires more analyte in order to obtain reliable data. For reproducible carbon isotope analysis about 12 ng carbon is necessary. For hydrogen isotope analysis 5 to 10 times more material is needed. Therefore, the efficiency of the extraction technique to enrich the target compound is critical for the lowest concentration that can be analyzed. Depending on the target, solvent extraction, solid phase micro-extraction, and purge & trap techniques have been shown to be appropriate for isotope analysis [44, 69].

The solvent extraction procedure, which is described in more detail elsewhere [34, 60, 86], was employed for the isotope analysis of BTEX and MCB in this work. Briefly, depending on the estimated concentrations, 1–2 mL *n*-pentane were used for the extraction of 1 L of groundwater. Although this extraction is not quantitative, no isotope effects were associated with this extraction method [16], and MCB and BTEX compounds can be analyzed to concentrations as low as some μg/L.

The beads of the microcosms were extracted using a dichloromethane-methanol-water mixture as a solvent modified according to Bligh et al. [9]. After phase separation, the dichloromethane phase containing the fatty acids (FA) was evaporated to dryness and derivated using a trimethylchlorosilane (TMCS) methanol mixture (1:8; v:v) as a reactant for 2 h at 70 °C to obtain fatty acid methyl esters (FAME) [79]. After evaporation to complete dryness, the FAME fraction was dissolved in *n*-hexane for subsequent analysis by GC-MS and GC-C-IRMS.

For the determination of concentrations and the identification or structural characterization of FAME and benzylsuccinic acid methyl ester by GC-MS, a Hewlett Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent Technology, Palo Alto, USA) was used. The carboxylic acid fraction was separated on a BPX-5

column (30 m × 0.32 mm × 0.25 μm; SGE, Darmstadt, Germany) with a temperature program of 120 °C initial temperature for 4 min, heat at 4 °C/min to 250 °C, heat at 20 °C/min to 300 °C, and hold for 10 min. FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco International) and quantified relative to an external standard mix or an internal standard (Henicosanoic acid, C21:0). The fatty acids are designated in the form of *A:B*ω*C* where *A* is the number of carbon atoms, *B* is the number of double bonds and *C* is the distance of the closest double bond from the aliphatic end of the molecule (unsaturation, ω-nomenclature. The prefix *i* (*iso*) and *a* (*anteiso*) refer to methyl branching. The benzylsuccinic acid methyl ester was characterized by co-injection and comparison with mass spectra obtained for the authentic reference compound which was derivated as described below.

The carbon isotope composition of the carboxylic acids fractions was analyzed using a GC-C-IRMS system. It consists of a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Bremen, Germany). The performance of the measurement has been described previously [60]. A BPX-5 column (50 m × 0.32 mm × 0.5 μm; SGE, Darmstadt, Germany) was used for chromatographic separation with helium as a carrier gas at a flow rate of 1.5 mL/min and a temperature program with an initial temperature of 60 °C for 2 min, heat at 20 °C/min to 120 °C, heat at 2 °C/min to 300 °C, and hold for 20 min [48]. The carbon isotope composition analysis of BTEX and MCB on the same system was described previously [34, 86].

The methylation of carboxylic acids for gas-chromatographic analyses introduces an additional carbon into the structure of the analyte, which affects its isotopic composition. Therefore the isotope signature of fatty acids ($\delta^{13}C_{FA}$) was corrected for the isotope effect due to derivatization FAME with methanol as described previously [3, 4, 21].

Molecular biological analyses of the beads from the exposed microcosms were performed according to the following procedure: For each sample, a total of 50 beads were transferred into 15 mL Falcon tubes containing 12 mL of TES buffer (50 mM $NaCl_2$, 10 mM Na_2EDTA, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride [Tris HCl], pH 8.0) with 1 % sodium dodecylsulfate (SDS). Five cycles of freeze-thaw lysis were performed, each with 5 min in liquid nitrogen (–196 °C) and 5 min at 65 °C in a shaking water bath, each cycle was interrupted by 10 s of vigorous vortexing. DNA was then extracted with phenol-chloroform, as described elsewhere [70, 71]. The isopropanol precipitated and purified DNA of each tube was resuspended in a total of 40 μL of 10 mM Tris in 10 μL aliquots. Aliquots were kept at 4 °C for further analysis or stored at –20 °C.

Partial 16S rRNA genes, including two variable regions, were amplified from total DNA by PCR, using primers Com1 and Com2ph [67]. The PCR products were converted into DNA-single strands by lambda-exonuclease digestion, following the protocol of Schwieger and Tebbe [71]. Genetic profiles of each sample were generated by single strand conformation polymorphism (SSCP) on non-denaturing polyacrylamide gels, as described elsewhere [17, 78]. Single bands were selected for further DNA-sequencing after PCR-amplification and cloning in *Escherichia coli*. Sequencing is described in the same references. Consensus sequences were compared to database sequences using the Fasta Nucleotide Database Query provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta).

3 Results and Discussion

3.1 Benzene and Toluene Degradation at the SAFIRA Reference Site Zeitz

3.1.1 Stable Isotope Fractionation Analyses (Cumulated Biodegradation Approach)

In order to obtain information about the degradation processes that have already been performed by the microorganisms at a contaminated site, the cumulated biodegradation approach was developed based on stable isotope fractionation analysis (SIFA). The overall workflow is summarized in Fig. 1. First, the concentrations of BTEX in the wells of the

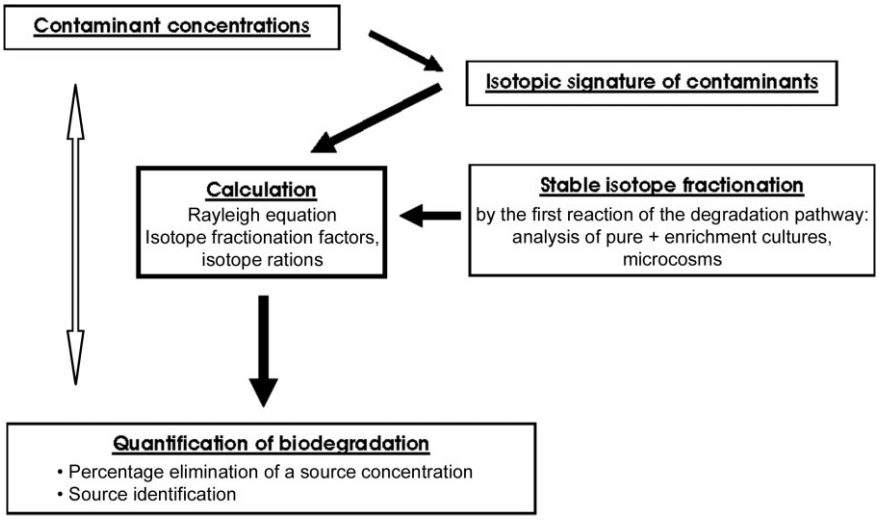

Figure 1. Approach I: Assessment of the cumulated biodegradation in a contaminated aquifer by use of stable isotope fractionation analysis (SIFA) at natural abundance; for details see text.

SAFIRA reference site Zeitz were analyzed where benzene is the predominant compound (see Fig. 2A). Next, the natural carbon isotope composition of benzene in the course of the plume was determined (see Fig. 2B). The center of the plume with concentrations of up to more than 950 mg/L was characterized by an average isotope composition of –28.5 ± 0.5 ‰. Down gradient, at the fringes of the plume, the concentration decreases to below 1 mg/L and the benzene isotope composition was enriched to maximum values of –24.4 ‰ indicating biodegradation [18].

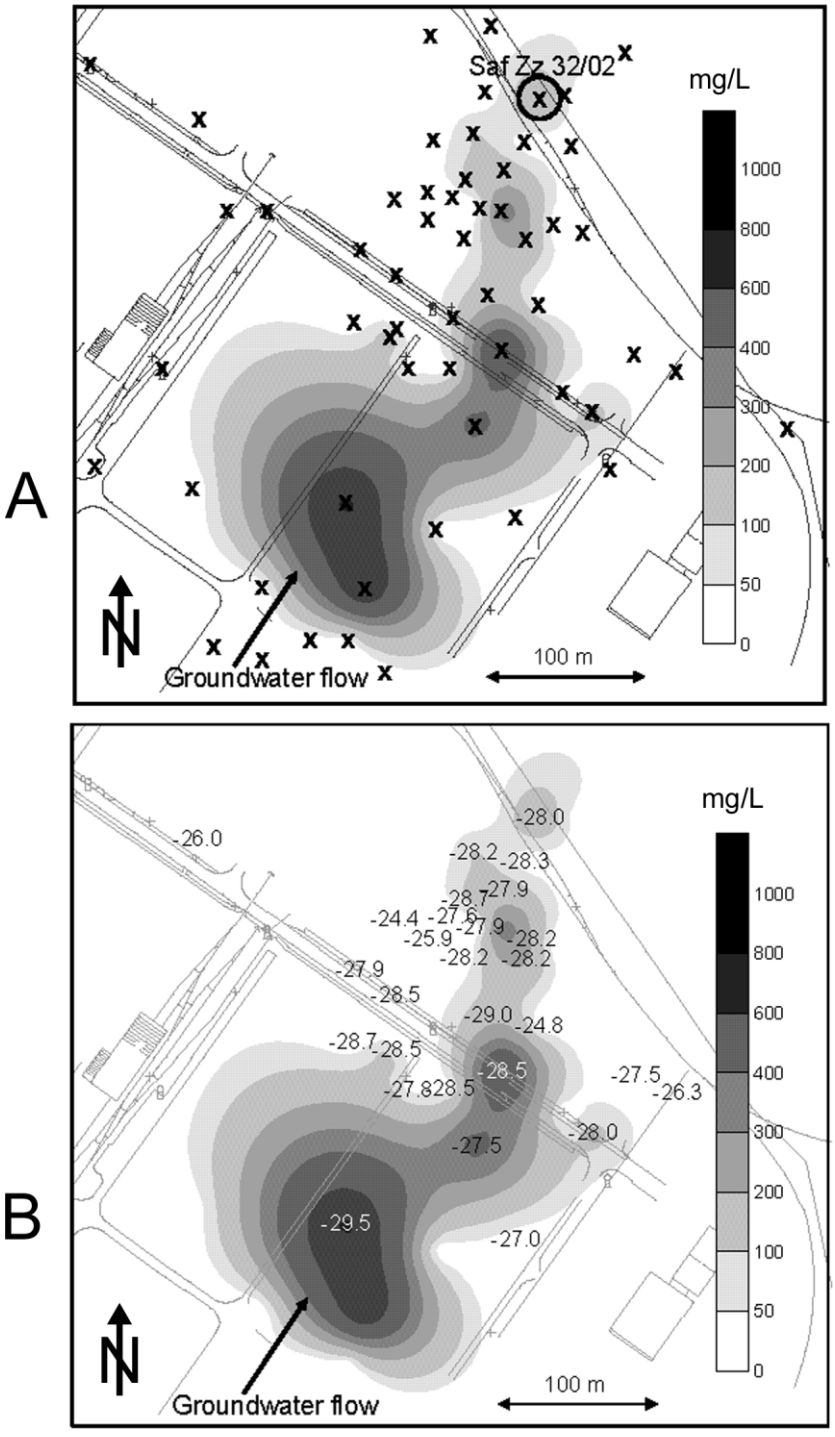

Figure 2. A) Spatial distribution of the benzene contaminations in the wells of the Zeitz aquifer in 2002 and 2004 (X = location of sampling wells; Saf Zz 32/02 well of the in situ microcosm experiment).
B) Spatial distribution of the carbon isotope composition of benzene in the wells of the Zeitz aquifer.

In order to quantify the extent of biodegradation, it is necessary to determine how a given microbial metabolic pathway fractionates $^{13}C/^{12}C$ during transformation. Isotope fractionation by microbial degradation is a result of the higher transformation rate of lighter isotopomers compared to heavier isotopomers by enzymatic reactions [8, 19]. This kinetic isotope fractionation enriches the amount of the ^{13}C isotopomer in the residual substrate. The isotope fractionation factor (α) describes the relation between the decrease in concentration and change in the isotope ratio during the degradation reaction and can be described by the Rayleigh equation (Eq. (2)). The isotope fractionation factor can be obtained in reference degradation experiments with pure cultures or at least well defined cultures in which the degradation pathway or the redox conditions are known and in which no other elimination process may alter the concentrations except biodegradation [44]. The extent of isotope fractionation depends on the initial enzymatic degradation reaction of the respective biochemical pathway. Without the knowledge of the terminal electron acceptor process and degradation pathway, isotope fractionation factors from microcosm studies are not very precise because they may vary upon experimental conditions. Finally, with an appropriate isotope fractionation factor, the percentage of biodegradation along a flow path in a contamination plume can be calculated by the data from the SIFA using Eq. (3).

The metabolic degradation pathway governs the extent of isotope fractionation and, therefore, it is important to apply the appropriate isotope fractionation factor for calculation. For example, from pure cultures able to degrade toluene it is known that the aerobic degradation and the resulting isotope fractionation can vary significantly depending on the initial oxidation reaction at the methyl group or at the aromatic ring system

[50]. However, under anaerobic conditions, the benzylsuccinate pathway attacking toluene at the methyl group shows quite similar isotope fractionation factors under nitrate- iron-, and sulfate-reducing conditions [26, 46, 49, 73, 93]. At a contaminated site, the microbial pathway relevant for the degradation can be deduced by the redox conditions in the aquifer or by assessment of the primary metabolites of the compound of interest [23]. In the case of toluene, benzylsuccinate was detected in Zeitz, which supports the assumption that sulfate reducing conditions govern the anaerobic degradation of toluene in the aquifer. Similarly, the fractionation of benzene also varies under these conditions (α = 1.0036) but is lower under methanogenic conditions (α = 1.002) [39]. Because sulfate was the major electron acceptor in Zeitz, here we used the fractionation factor for benzene degradation under sulfate reducing conditions for calculating the in situ degradation. To show the uncertainty, we also calculated the degradation with a fractionation factor characterizing methanogenic conditions (α = 1.002). Using the larger fractionation factor (α = 1.0036) characteristic for sulfate reducing conditions, the interpretation of the benzene isotope composition gives a more conservative estimate (up to 69 % degradation) whereas the calculation with the lower factor (α = 1.002) indicative for methanogenic conditions suggests a higher degradation (up to 88 %). A field survey over the contamination plume enabled mapping of the degradation of contaminants (see Fig. 3). A previously published monitoring campaign in Zeitz shows similar results [86]. Examinations of other wells or other BTEX contaminated sites are described elsewhere [10, 20, 76, 77].

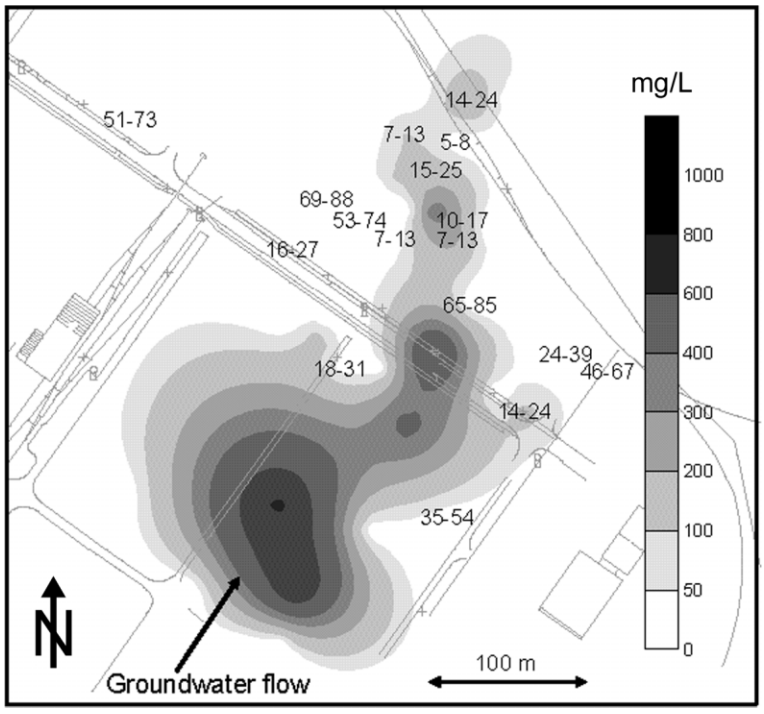

Figure 3. Percentage of benzene degradation in the wells of the contamination plume calculated by use of Eq. 3 (isotopic composition of the source [R_0] = –28.5 ‰, α = 1.0020 (methanogenic) –1.0036 (sulfate reducing); modified from [18]).

In addition, a number of field studies exist where the enrichment of heavy isotopes in the residual fraction of mostly BTEX or chlorinated ethenes was used to estimate the in situ degradation [13, 18, 23, 36, 45, 51, 56, 60, 62, 63, 75, 85–87]. For other compounds such as naphthalenes, chlorinated benzenes, phenols, fuel oxygenates (MTBE, ETBE, [95]) less experience exists showing the need for further field studies. The rapid increase of the references within the last five years shows a broad acceptance of the general approach, and the reliability of the technique will be further improved with increasing data sets for isotope fractionation factors. At present, a range of fractionation factors for several compounds under different environmental conditions are reported in recent reviews [47, 69] and data bases (www.isodetect.de). Particularly for the mono-aromatic BTEX contaminants, the applicability of the technique is highly reliable. However, at present the cumulated biodegradation or SIFA approach is of limited value for contaminants such as tert-butyl alcohol, tri-, dichloroethylene, and vinylchloride which can be both, substrate or metabolite. Aquifers contaminated by tetra- and trichloroethene may show a sequential transformation pattern with varying isotope compositions which are not easy to interpret [42].

3.1.2 In Situ Microcosms with Stable Isotope Labeled Substrates (BACTRAP Approach)

At many sites, however, there is only limited access to the contamination plume due to the small number of wells or the insufficient data base for the respective compounds. Therefore, a method to prove the microbial degradation of the contaminants is necessary which can be applied directly in the groundwater monitoring wells. In order to provide evidence and to estimate the turnover and the relevant pathway, the in situ microcosm approach was developed [20]. The system consists of beads containing activated carbon in a granular shape that are loaded with ^{13}C-labeled contaminants as shown for benzene or toluene at the Zeitz site. The beads provide a large amount of internal surface area for colonization or attached growth of degrading bacteria (biofilm formation). If the indigenous bacteria of the well colonize the beads and productively consume the contaminants under in situ conditions, the ^{13}C-labeled carbon will be incorporated into the growing cells and can be traced within biomarker molecules such as fatty acids or nucleic acids. The overall workflow of the approach is presented in Fig. 4.

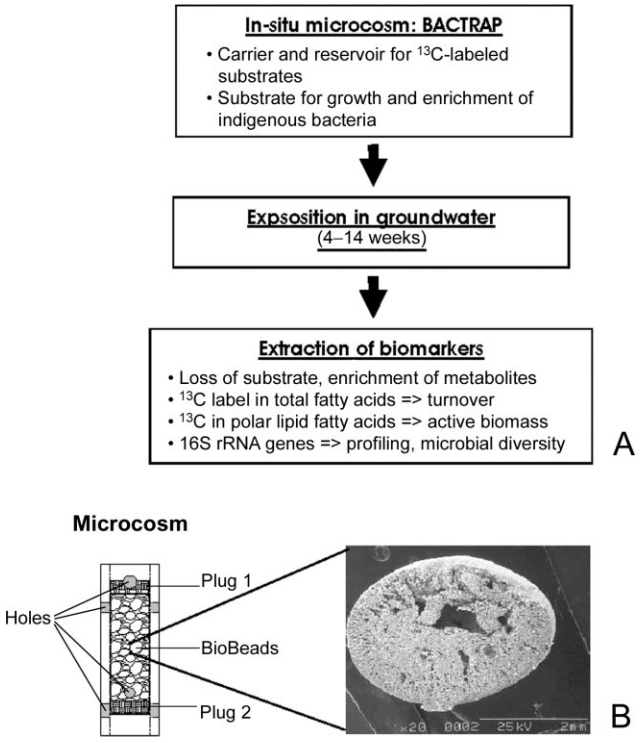

Figure 4. Approach II (A): Assessment of biodegradation potentials in a contaminated aquifer by use of in situ microcosms (BACTRAPs) with tracer compounds labeled with stable isotopes; B: microcosm containing Bio-Sep® beads.
(Picture: Copyright (2003) from Environmental Forensics, 2003, 4 (1) by White et al.; reproduced by permission of the Taylor & Francis Group, LLC., http://www.taylorandfrancis.com).

In order to analyze the microbial activity, sterilized in situ microcosms were loaded with ^{13}C-labeled benzene or toluene and were exposed for 32 d in well Saf Zz 32/02 in Zeitz. Non-amended microcosms served as controls. After exposure and development of the biofilms on the beads, the residual substrates and the lipid biomarkers were extracted and analyzed by means of mass spectrometry. About 88 to 112 mg of benzene and toluene per g of bead material in the microcosm was initially applied and around 16 mg/g was recovered after exposure (see Tab. 1). Isotopic analysis of the residual amounts of ^{13}C-benzene or ^{13}C-toluene in the recovered microcosms from Saf Zz 32/02 in September 2003 showed only a slight accumulation of the contaminants from the aquifer in the bead material in this well. In July–August 2004, however, an accumulation of up to 5.6 mg/g from the aquifer was observed in the controls after 112 d of exposure as a result of the much higher contaminant concentrations of about 300 mg/L at this time. The overall standard deviation of the recovered substrates from the BACTRAP systems amounted to < 10 %.

Total lipid fatty acid (TLFA) patterns of extracts and their isotopic compositions, as well as polar lipid fatty acids (PLFA) of the beads from the microcosms were analyzed and the TLFA patterns are shown in Fig. 5. TLFA from

Table 1. Concentrations and ^{13}C abundance of contaminants in the in situ microcosms (BACTRAPs) before and after exposure in well Saf Zz 32/02 (modified from [20]).

	Before exposure		After 32 d		
	^{13}C	Load	^{13}C	Concentration	Loss
	[Atom%]	[mg/g [a)]]	[Atom%]	[mg/g[a)]]	[%]
Benzene	0.1	88 ± 1.0	0.1	15.8 ± 0.2	82
Toluene	0.1	112 ± 1.1	0.1	16.0 ± 1.4	85
[$^{13}C_6$]-Benzene	98.0	88 ± 0.2	98.0	15.8 ± 0.2	82
[$^{13}C_1$]-α-Toluene	14.0	112 ± 1.2	14.0	17.8 ± 1.4	84
Control	–	0.0	0.1	0.46 (benz.)[b)]	–
(Control)	–	0.0	0.1	5.6 (benz.)[c)]	–

[a)] Per g bead material.
[b)] Non-loaded control; accumulation from aquifer contaminant plume after 32 d of exposure in September 2003.
[c)] Non-loaded control; accumulation from the aquifer after 112 d of exposure in July–August 2005 showing much higher benzene concentrations and lower biofilm formation.

both, ^{13}C-benzene and ^{13}C-toluene amended microcosms showed ^{13}C-enrichment of up to δ = 13360 ‰ in the fatty acids, providing clear evidence for benzene and toluene biodegradation with transformation of ^{13}C into the microbial biomass. The decrease in contaminant concentrations of the microcosms can thus be considered to be attributable to microbial activity. Extracts from ^{13}C-toluene-amended beads showed incorporation of ^{13}C into more or less the same fatty acids as found in the ^{13}C-benzene microcosm.

Estimation of the ^{13}C Recovery in the Biomass

The incorporation of ^{13}C into the fatty acid fraction causing a shift of up to δ 13360 ‰ indicates that [^{13}C]-α-toluene (~14.5 atom %, see Tab. 1) was more intensively incorporated into microbial biomass than [$^{13}C_6$]-benzene (98 atom %). This was expected because toluene is considered to be more easily degradable than benzene. For 16:1ω7c, the isotope composition was 13360 ‰ (13.9 atom %), very similar to the toluene substrate, which indicates that at least one organism producing this fatty acid used the applied toluene nearly exclusively as a carbon source. The isotope signatures of C15:0, C16:0 and 18:1ω7c, of 7946 ‰ (9.1 atom %); 6283 ‰ (7.6 atom %); and 9684 ‰ (10.7 atom %), respectively, demonstrate that other organisms in the consortium assimilated the labeled C from toluene to a major extent but not exclusively. Fatty acids with a lighter isotope composition such as for 18:0 with 92 ‰ (1.2 atom %) indicated that some organism colonized the in situ microcosms using other carbon sources than the labeled toluene, e.g., other contaminants or natural organic matter to a major extent.

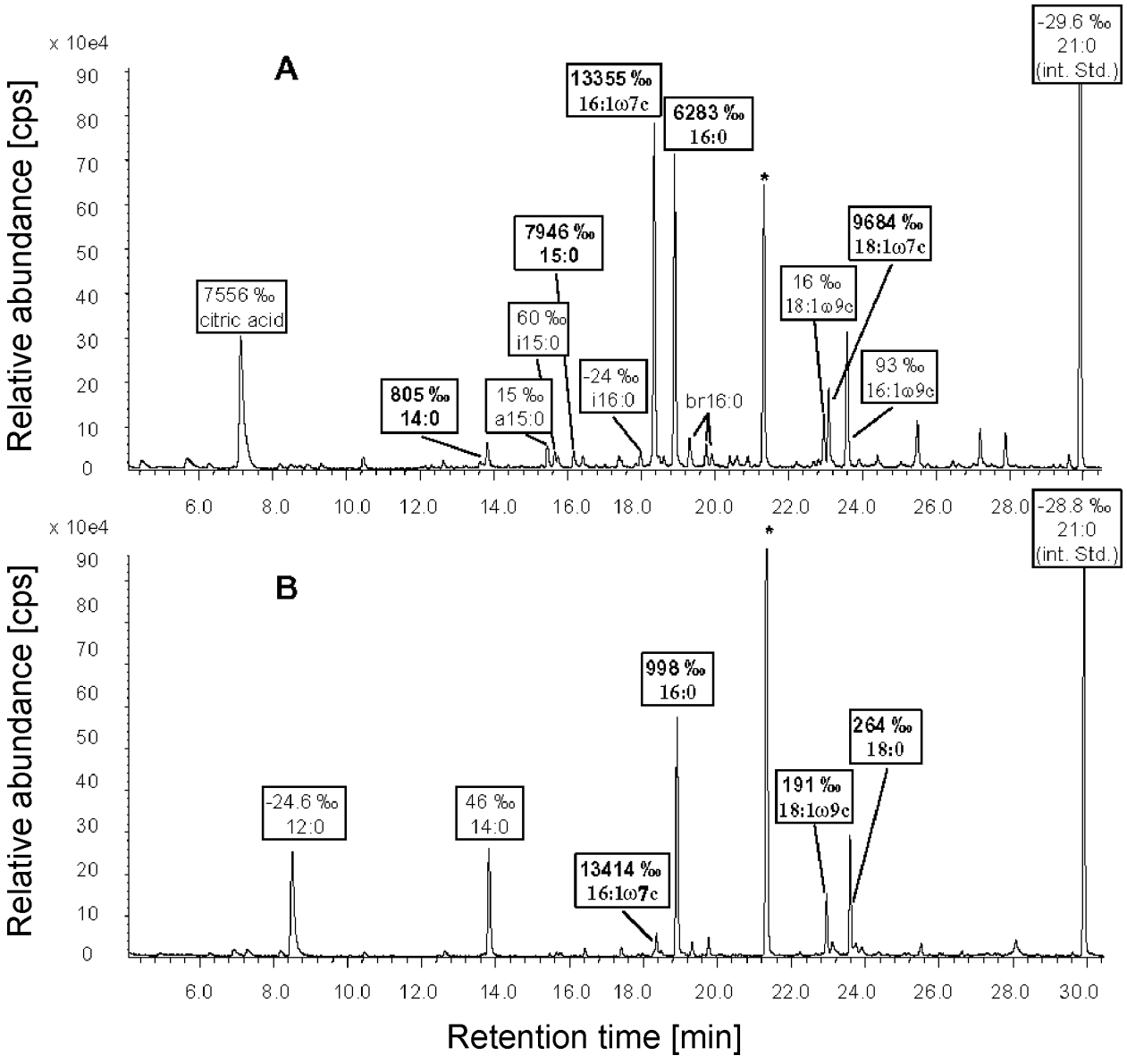

Figure 5. GC-MS profiles and isotope enrichment of fatty acids in total lipid extracts obtained from the [$^{13}C_1$]-α-toluene (A) and [$^{13}C_6$]-benzene (B) amended microcosms deployed in well Saf Zz 32/02 in the Zeitz aquifer for 32 days.
Major fatty acids with retention time in brackets given in minutes were 14:0 (13.83), i15:0 (15.46), a15:0 (15.66), 15:0 (16.42), i16:0 (17.98), 16:1ω7c (18.36), 16:0 (18.91), 18:1ω9c (22.96), 18:1ω7c (23.10), 18:0 (23.59), br16:0 (19.92, 20.41, 20.60) with undetermined position of methyl-branching, * phthalate (21.31) from extraction process.
(The δ ^{13}C values for the isotopic composition of the respective methyl esters represent the isotopic shift compared to the PDB standard.)

The most intensively labeled fatty acid on the [$^{13}C_6$]-benzene amended microcosms was 16:1ω7c with an isotope composition of 13410 ‰ corresponding to 13.9 atom %. The isotope composition of [$^{13}C_6$]-benzene was about 98 atom % and therefore around $^1/_7{}^{th}$ of the carbon was derived from the labeled benzene. Thus, the majority of carbon was derived from non-labeled carbon sources which may comprise non-labeled benzene from the contamination, natural organic carbon sources or CO_2 used for synthesis of fatty acid. The C16:0 and C18:0 fatty acids were much lower labeled indicating that some organisms use benzene only to a minor extent for biosynthesis. Almost non-labeled fatty acids (C12:0) suggest that parts of the microbial community colonizing the beads are not involved in the biodegradation of benzene and use other carbon sources exclusively.

The concentrations and isotope compositions of individual fatty acids were used to calculate the amount of ^{13}C converted from the contaminant according to the method described by Richnow et al. [61]. Since the isotope composition of fatty acids from the non-amended in situ microcosms was typically between –35 and –25 ‰, it was considered that an isotope composition enriched to more than –20 ‰ was signif-

icantly affected by the transformation of the labeled contaminants. On the benzene amended trap about 3.1 nmol ^{13}C/g bead material corresponding to 43 ng ^{13}C/g labeled benzene was bound in the fatty acid fraction. The ^{13}C-*a*-toluene derived carbon bound within fatty acids was orders of magnitude higher. The amount of 456 nmol toluene derived carbon/g corresponds to the equivalent of 6062 ng labeled toluene. Therefore, the assimilation of C from toluene was more than 140 times higher than that of benzene. Considering that fatty acids account for up to 5 % of the total biomass carbon and that the carbon isotope signature in lipids is similar to that in other fractions of the biomass, the overall amount of labeled carbon bound within the biomass is 20 times higher than that found in the fatty acids. If we expect furthermore, that around 95 % of the carbon source is metabolized and 5 % is used for biosynthesis of biomass ($y_{x/s}$ = 0.05), the total productive metabolization can be estimated as 2400 μg toluene and 17.2 μg benzene during incubation in the aquifer. These values only account for 2.5 % of the toluene loss and 0.024 % of the benzene loss. The ^{13}C-label of benzene, however, was highly diluted due to the background concentrations of benzene from the aquifer.

Phospholipid fatty acids (PLFA) are parameters for microbial membrane phosphoglycerolipid content and thus an indicator for viable cells [24]. The total PLFA amounts were highest in the ^{13}C-toluene microcosms with 8870 pmol and 8480 pmol in the ^{12}C-toluene microcosm [20]. The microcosms with ^{13}C- or ^{12}C-benzene and the control contained 5860 pmol, 2410 pmol and 860 pmol PLFA, respectively. Applying the conversion factor of $1.4–4.0 \times 10^4$ cells/pmol of PLFA, these values correspond to $2.3 \times 10^7–2.4 \times 10^8$ bacterial cells with the size of *Escherichia coli* [91] verifying a considerable microbial colonization in the bead material of the microcosms.

Even though no strictly anaerobic pure cultures able to degrade benzene under iron or sulfate reducing conditions are yet available, the in situ microcosm approach with ^{13}C-labeled compounds could prove the microbial in situ degradation of benzene under strict anaerobic conditions in Zeitz. At present, only nitrate or perchlorate reducing pure cultures responsible for the degradation of benzene have been described [11]. The transformation of ^{13}C-carbon from the labeled substrate into microbial fatty acids provided clear evidence for the assimilation of the pollutant resulting in the formation of biomass. This easy to handle and cost-effective microcosm approach can be used to provide the information required to implement Monitored Natural Attenuation (MNA) as requested by the OSWER directive [80] for approval of Natural Attenuation as a treatment measure for contaminated sites.

Metabolites

The activated carbon of the beads may enrich degradation metabolites that can be used to identify the involved degradation pathways. The formation of labeled benzylsuccinate upon toluene degradation was observed in the Zeitz aquifer (data not shown) and is indicative of the anaerobic degradation pathways of aromatic compounds in microorganisms using nitrate, sulfate, and iron as terminal electron acceptors [26, 73, 93]. The non-labeled analogue was also found in the control microcosms indicating a degradation of the aquifer contaminants under the respective conditions. Reusser et al. [59] applied push and pull experiments with deuterium labeled toluene and xylene to verify in situ degradation of the compounds in a contaminated aquifer. Although deuterium labeled contaminants may be useful tracer compounds for proving in situ degradation, much higher amounts of labeled substances are needed during push and pull experiments compared to the BACTRAP approach. The application of in situ microcosms as described here can significantly reduce the amount of labeled material and gives, in principle, information similar to that obtained from push and pull experiments. However, by using ^{13}C-labeled compounds in the in situ microcosms the transformation into biomass can be traced by analyzing the biomarker molecules providing the proof of principle for biodegradation.

Biodiversity

The biodiversity of the microbial biomass from in situ microcosms exposed in well Saf Zz 32/00 in summer 2004 over a period of 7 to 98 d was characterized by cultivation independent molecular biological approaches based on PCR-amplified partial 16S rRNA genes from total DNA [72]. Total DNA was directly extracted from the in situ microcosms and the PCR products amplified from the various samples were compared to each other on the basis of their SSCP profiles (see Fig. 6). The community profiles were typically composed of approx. 20 bands with about 5 to 10 bands being more dominant. Most of the profiles generated from in situ microcosms taken after different incubation times were relatively similar to each other, indicating a highly reproducible colonization pattern of the bead material. For most samples analyzed, the compositions of the bacterial community on the benzene and toluene loaded microcosms were not severely altered in comparison to the non-amended controls although highly labeled microbial fatty acids extracted from the ^{13}C-experiments showed a substrate degradation and incorporation into biomass (see Tab. 1). This indicates a coordinated degradation by certain members of the indigenous microbial community colonizing the in situ microcosms and channeling of the ^{13}C carbon into the microbial food web. However, the isotope composition showed that these organisms also used other carbon sources. Benzene and toluene were the major carbon sources but other BTEX compounds, contaminants, or humic substances were certainly present and may be used as additional carbon sources.

The comparison of the profiles of the non-amended controls with the loaded microcosms showed that the DNA and

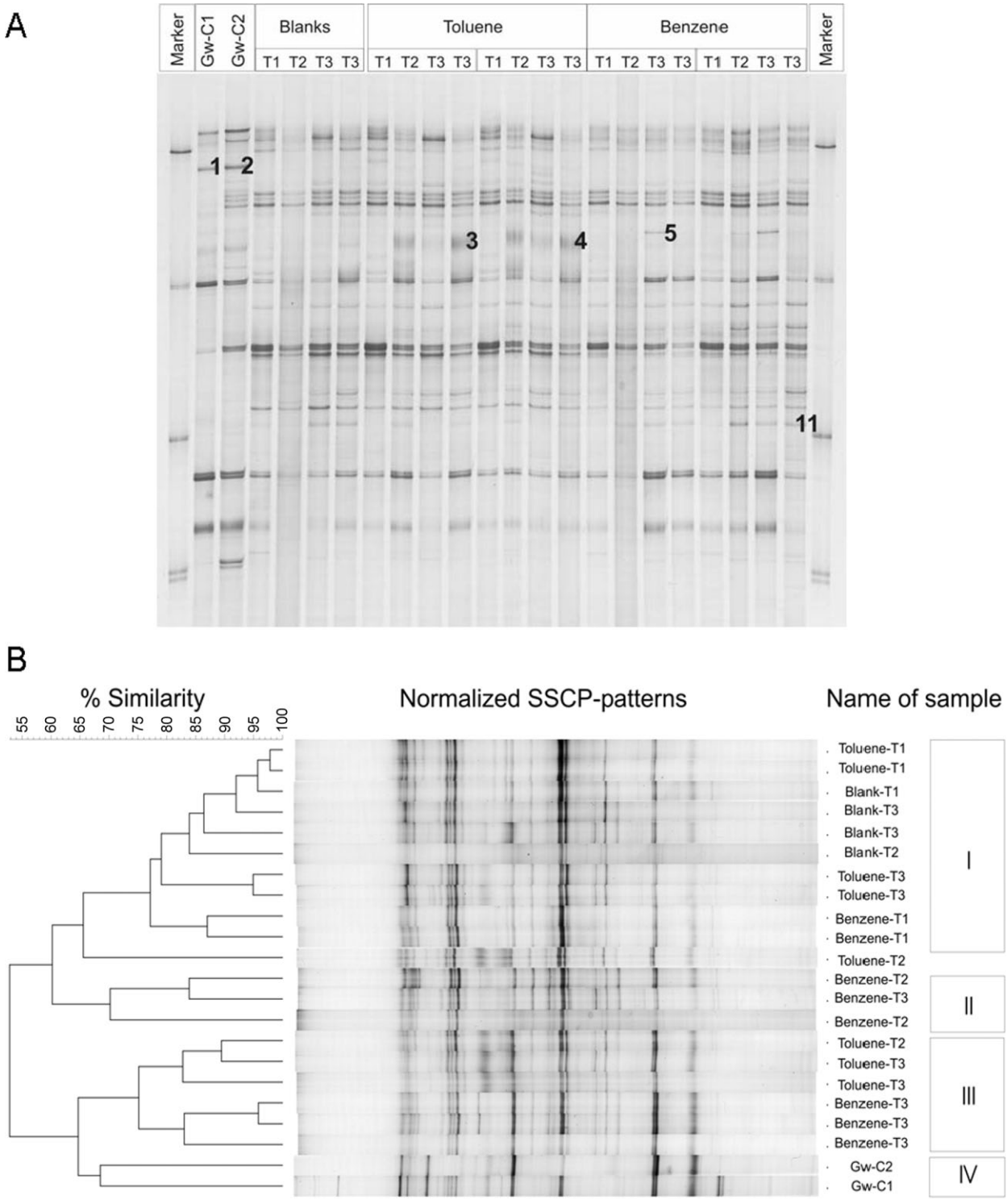

Figure 6. SSCP Gel (A) and digital image analyses of SSCP-profiles (B) of PCR amplified 16S rDNA isolated from groundwater and microcosms loaded with toluene or benzene and controls (blanks) after exposure of 24 d (T1), 49 d (T2), and 92 d (T3) in well Saf Zz 32/02.
Selected bands of these profiles were sequenced for identification, numbers indicated in the gel image can be found in Tab. 2.

FA patterns were only slightly different. This reflects the fact that the bead material (activated carbon) of the control (blank) extracted a certain amount of contaminants, in particular, benzene from the contaminated aquifer (see also Tab. 1) leading to similar conditions in the non-loaded and benzene-loaded microcosms. A comparison of the SSCP-profiles of the bead samples with the profiles of the groundwater samples from the same well showed that with the ex-

ception of two bands all bands found in the water were also present on the beads; conversely, only three bands found on the beads were not present in the groundwater samples (see Fig. 6A). Hence, it can be concluded that the bead material provided a nearly non-selective surface for the growth of the indigenous microbial community from BTEX contaminated groundwater.

DNA-patterns from the toluene loaded microcosms were also highly similar indicating that toluene degraders were already members of the colonizing community and may have conducted the toluene degradation at the site. Cluster analysis of the profiles (see Fig. 6A) revealed four major groups (see Fig. 6B). Cluster I contained communities from all microcosms collected after short time exposure (24 d) or without loaded substrates (T1, see also legend of Fig. 6). In addition, two toluene loaded microcosms from a later sampling (T3) were found in this group. Cluster II contained samples from microcosms loaded with benzene. Other replicates of this treatment and sampling were found in Cluster III which mainly contained samples from substrate loaded treatments, collected later during the kinetic study. As expected, on the one hand, the community profiles generated from the surrounding groundwater was different from the biofilm material extracted from the beads (Cluster IV), but on the other hand, similarities between both sample types were evident. This indicated that, in fact, the dominant bacteria from the surrounding groundwater contributed to the biofilm communities. Interestingly, the similarity of the groundwater samples was higher with Cluster III (later samplings) than that with Cluster I, which can be explained by the fact that several of the bacteria from the aquifer were only slowly growing on the bead material.

Due to the similarity of the profiles to each other, it was in most cases not really possible to identify bands which were highly characteristic for a certain substrate or period of incubation. However, for toluene, a characteristic band seems to be generated during the incubation (see Fig. 6, bands 3 and 4). DNA-sequencing of selected bands revealed that most bacteria in this community were Proteobacteria from the Alpha- and Beta-subgroup (see Tab. 2). Similarities to known sequences in the databases for these partial rRNA genes ranged from 85 to 100 %. Complete similarity was found with an uncultured bacterial clone isolated from monochlorobenzene contaminated groundwater [5]. Other sequences, which were derived from the toluene-specific bands, were found to originate from *Azoarcus* sp., one of them most closely related to an organism that has been shown to degrade toluene and *m*-xylene under denitrifying conditions [7, 94]. Despite the fact that 16S rRNA genes are generally not reliable indicators for bacterial functions, the so far characterized sequences give no rise to assume growth of sulfate-reducing or other strictly anaerobic bacteria, but rather indicates the presence of bacteria capable of growing under denitrifying conditions. Other sequences indicated α-proteobacteria adapted to the exposition with metals, assuming physiological similarities with their closest relatives [37, 74]. However, it is known from other strictly anaerobic bacteria, for example, *Dehalococcoides* sp. that no PCR products were found with the universal COM1/COM2 primers.

3.2 Monochlorobenzene Degradation at the SAFIRA Reference Site Bitterfeld

3.2.1 Stable Isotope Fractionation Analysis (Cumulated Biodegradation Approach)

Monochlorobenzene (MCB) is a xenobiotic compound which has been commonly used for chemical manufacturing processes over decades and is a common groundwater contaminant at many chemical production sites in Europe and the United States [6, 65]. In addition, higher chlorinated benzenes can be degraded via reductive dechlorination to MCB under anaerobic conditions (for review see Van Agteren et al. [82]) and may also be produced from the microbial trans-

Table 2. Characterization of rRNA genes isolated from groundwater and microcosms loaded with toluene or benzene and controls after exposure in well Saf Zz 32/02 (see also Fig. 6).

Band No. [a]	Length [bp]	Similarity [%]	Closest relative or sequence (Accession No.)	Phylogenetic group	Source of isolation or habitat of closest relative or relevant metabolic activity of closest relative
1	370	100	Uncultured bacterium (AY05094)	Betaproteobacteria	Monochlorobenzene contaminated aquifer
2	205	85	*Acidovorax* sp. (AJ864847)	Betaproteobacteria	High mountain lake habitat
3	370	99	*Azoarcus* sp. (AF515816)	Betaproteobacteria	Degradation of toluene and benzene under denitrifying conditions
4	360	95	*Azoarcus denitrificans* (L33690)	Betaproteobacteria	Growth on toluene under denitrifying conditions
5	350	93	Uncultured bacterium (AY475201)	Alphaproteobacteria	From metal rich, acidic river Tinto (Spain)
11	210	90	Uncultured bacterium (AF418953)	Alphaproteobacteria	From metal rich freshwater reservoir

[a] PCR-products obtained from bands indicated in Fig. 6A; no products were obtained from other bands.

formation of hexachlorocyclohexane (HCH) in anoxic aquifers [57, 83]. MCB is the major contaminant of the pollution plume in the Quaternary anoxic aquifer in Bitterfeld. The aquifer belongs to the regional aquifer system, in which about 200 million m^3 of groundwater are contaminated with predominantly chlorinated compounds [27, 28]. Field studies demonstrating the anaerobic microbial transformation of MCB are completely lacking. Thus, no pathways and mechanisms of anaerobic transformation of MCB are yet known. Taking into account that the plume formed in the aquifer several decades ago, a significant selection pressure for MCB degrading microorganisms can be expected. Therefore, assuming biodegradation we applied the SIFA approach (see Fig. 1) to the complex contamination plumes in Bitterfeld [34].

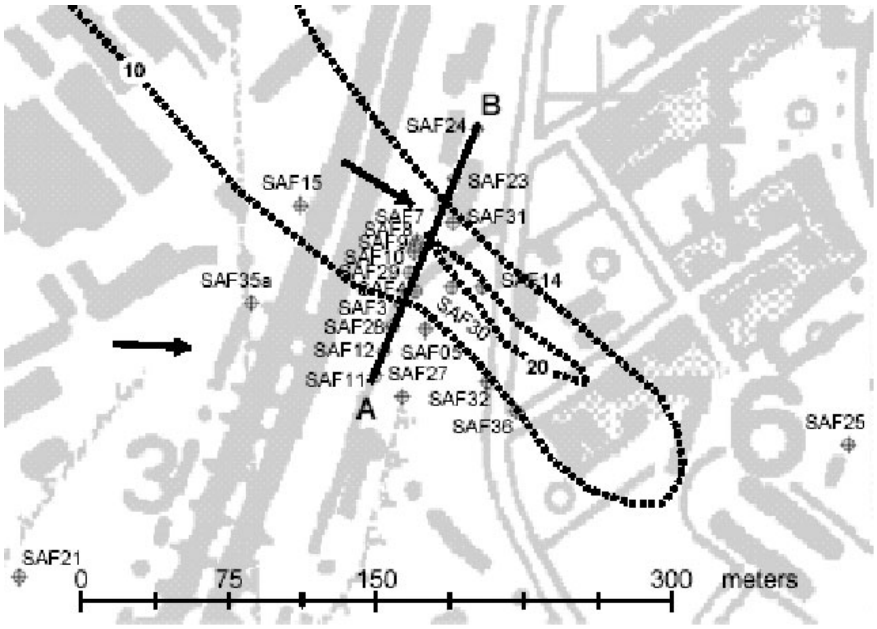

Figure 7. Monitoring wells and distribution of the monochlorobenzene plume in Bitterfeld modified from [35]. Concentrations are given by the dotted lines in mg/L.
The arrows indicate the groundwater flow in November 2002. The solid line (A to B) represents the position of the cross-section perpendicular to the monochlorobenzene plume at the location of the SAFIRA research station. (Saf 4 = monitoring well for the vertical analysis).

The MCB plume stretches downgradient in a SE-direction, following a Quaternary hydrogeological channel structure oriented in a NW/SE direction, which strongly affects contaminant flow. Since the access to the groundwater by wells was limited, we decided to analyze a cross section of wells A-B perpendicular to the groundwater flow in the lower aquifer which covers the concentration gradients at both sides of the plume (see Fig. 7). The concentrations of MCB increased up to 20 mg/L in the center of the plume and the isotopic signatures increased from values of –26.7‰ at the center to –23.0‰ at the fringes of the plume (see Fig. 8 left side) showing a significant ^{13}C-enrichment in the residual MCB at the fringes. In addition, we analyzed the vertical distribution of MCB and its isotope signature in the upper aquifer by multilevel sampling in well SAF 4/97 showing the same trends with a clear vertical MCB stratification (see Fig. 8, right side). The ^{13}C-enrichment found at the fringes of the plume relative to the source area of about 4 δ units clearly shows the occurrence of in situ biodegradation of MCB. This would result in a field isotope fractionation factor of $\alpha = 1.0005$. However, field fractionation factors are always

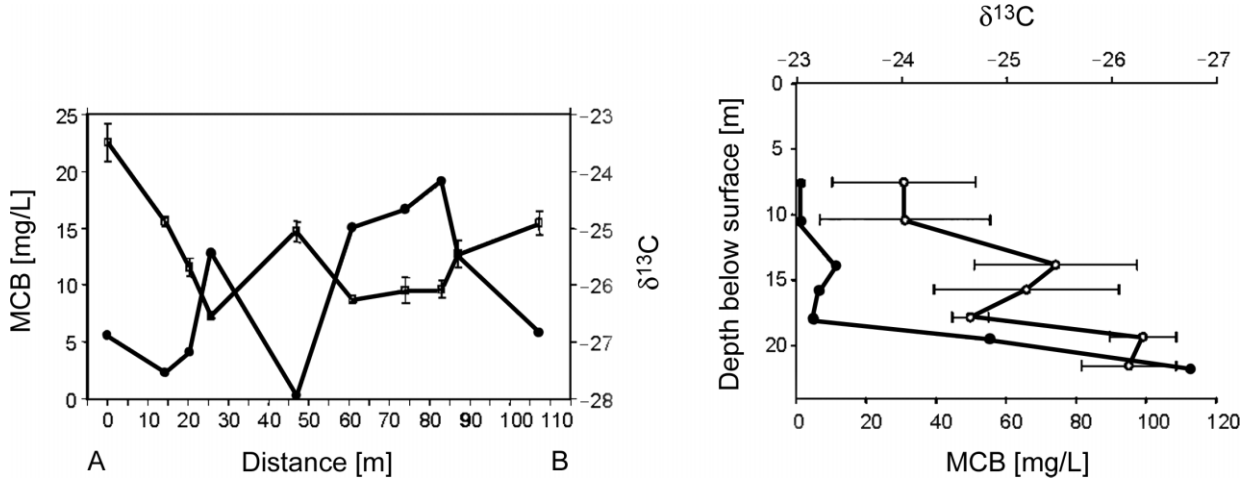

Figure 8. Monochlorobenzene concentrations and isotopic signatures of the cross-section A–B, depth of 17–21 m (left) and the vertical profile in Saf 4 (right); modified from [35].
● = MCB concentrations □ and ○ = $\delta^{13}C$ of MCB.

lower than factors obtained from laboratory experiments in closed systems in which only biodegradation can diminish the concentrations. In the field, the concentrations may be affected by abiotic effects leading to much smaller fractionation factors suspending the calculation of the effective biodegradation according to Eq. (3).

Several degradation pathways are conceivable under anaerobic conditions. MCB may be degraded similarly to other aromatic compounds by processes such as nitrate-, Fe(III)-, sulfate-reduction, or fermentation coupled to methanogenesis [11, 26, 73, 81, 93]. In addition, microbial reductive dechlorination of MCB requiring an electron donor may also be considered. Batch experiments using several aerobic pure cultures of bacteria showed that the known aerobic pathway initiated by dioxygenases did not result in a significant carbon isotopic fractionation [34]. Conversely, the field isotope fractionation factor for MCB obtained in the anoxic aquifer was higher, especially, if dispersion and dilution effects are accounted for [34]. Thus, it is evident that in this aquifer a novel anaerobic pathway resulting in an isotopic fractionation, which is untypical for aerobic MCB transformation, must be considered to be the predominant process of the in situ MCB degradation. However, the present data do not allow the anaerobic pathway of MCB degradation to be identified. The transformation of MCB appears to be very slow and most of the microbial activity appears to be located at the plume fringes.

Fig. 9 illustrates the various fractionation processes likely for the MCB degradation in comparison to the field data. Since these field-derived fractionation factors for MCB are significantly higher than the laboratory-derived factors for aerobic degradation, it is evident that an anaerobic pathway governs the MCB fractionation in the Bitterfeld aquifer. All

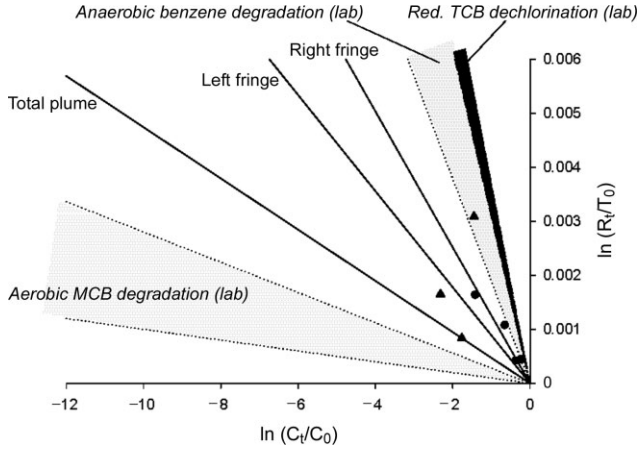

Figure 9. Rayleigh plot illustrating field-derived and laboratory-derived fractionation factors: aerobic degradation of MCB in batch experiments (α = 1.0001–1.00028), the field fractionation factor for the whole MCB plume (α_f =1.00043), the (▲) left (Saf11, Saf12, Saf27, Saf28) and (●) right fringe (Saf7, Saf30, Saf31, Saf23) of the plume at the cross-section A–B (α_f = 1.00083 and 1.00106, respectively) and, for comparison, batch experimental data for the reductive dechlorination of TCBs (α =1.0031–1.0037 [22] and anaerobic degradation of benzene (α =1.0019–1.0036 [39]).

known aerobic microbial cultures investigated used the ring-dioxygenase pathway to degrade MCB [88] but the dioxygenase pathway did not lead to a significant isotope fractionation of MCB in batch experiments. This result is in accordance with studies using toluene and naphthalene as model compounds showing that the dioxygenase reaction does not lead to significant carbon isotope fractionation [50]. Aerobic degradation of TCB by pure cultures exhibiting the dioxygenase reaction also did not show any significant isotope fractionation [22]. However, the field fractionation factors obtained are lower compared to laboratory experiments investigating the anaerobic degradation of benzene or 1,2,3-trichlorobenzene (TCB) [22, 39]. This may be explained by dilution, dispersion and sorption processes in the aquifer, which affect concentrations (C_t) without altering the isotopic composition (R_t). Hence, when using the Rayleigh equation (Eq. (2)) for field data, the resulting field isotope fractionation factor is always lower than that determined in culture experiments. However, in a cross section perpendicular to the contaminant plume, the dispersion and sorption processes should have affected MCB concentrations in a similar way, since the distance from the original source of the contamination is comparable. Accordingly, the fractionation factor determined at the left and right fringe was higher than that of the whole plume and was significantly higher than the low isotopic fractionation expected from aerobic transformation. The anaerobic degradation of various aromatic hydrocarbons led to a significant isotope fractionation of about α = 1.0017 to 1.0036 [39, 46, 49].

In addition, a number of studies have demonstrated that the anaerobic reductive dehalogenation of halogenated ethenes and TCBs is always associated with higher isotope fractionation factors [22, 23, 32, 33]. Analogously, it can be expected that a reductive dehalogenation of MCB should also be associated with a significant isotope fractionation. Alternatively, MCB may be degraded similarly to benzene under anaerobic conditions. Although the anaerobic degradation pathway for benzene has not yet been completely identified, the isotope fractionation of benzene yields fractionation factors of 1.0020 (methanogenic), 1.0036 (sulfate-reducing) and 1.0022–1.0024 (nitrate-reducing conditions) in enrichment cultures [39]. A degradation of MCB following the anaerobic benzene pathway should therefore also show a significant isotope fractionation.

3.2.2. In Situ Microcosms with Stable Isotope Labeled Substrates (BACTRAP Approach)

The in situ approach based upon microcosms amended with ^{13}C-labeled tracer compounds was also applied for MCB in the aquifer in Bitterfeld. The accumulation of MCB in anoxic aquifers initially led to the assumption that anaerobic biodegradation of MCB is difficult or even impossible, because neither pure nor enrichment cultures and microcosm studies had demonstrated biodegradation of MCB un-

der these conditions. However, the isotopic signatures of the contamination plumes clearly show biotransformation in the course of the MCB plume [34]. BACTRAPs amended with MCB were incubated for 7 weeks in a groundwater well under anoxic conditions. Geochemical analysis led to the conclusion that anaerobic conditions prevailed in the well where the BACTRAPs were tested.

After exposition for 49 d, more than 80 % of ^{13}C-MCB had been lost from the microcosms and the extracted total lipid fatty acids were found to be significantly labeled (see Fig. 10). Hexadecanoic acid (16:0) showed the highest incorporation of ^{13}C with δ^{13}C of 579 ‰ (see Fig. 10). Octadecenoic acid (18:1) was also labeled significantly to 158 ‰. Octadecanoic acid (C18:0) and tetradecanoic acid (14:0) showed a lower incorporation of ^{13}C of 10 ‰ and 46 ‰, respectively. Unsaturated hexadecenoic acid (16:1) showed a strong ^{13}C signal indicating high label incorporation but the concentration was also too small for quantitative analysis. Similar to the in-situ microcosm studies with benzene and toluene, the data provided evidence for the assumption of an unknown anaerobic MCB degradation process actually occurring in Bitterfeld. In addition, the incorporation of the label into marker molecules of the biomass also clearly indicates that this pathway supports growth and energy conservation from MCB for the respective bacteria under the given environmental conditions.

4 Concluding Remarks

Both approaches presented in this overview provide promising tools for the assessment of in situ microbial degradation activities in contaminated aquifers. They have certain implications and the evaluation of the practical benefits and limitations is currently in progress. The isotope fractionation (SIFA) approach interpreting the isotope signatures of contaminants provides a technique for assessing the cumulated biodegradation in an aquifer, which has occurred on the groundwater flow path down gradient from a source of contaminants. SIFA relies on the availability of a sufficient number of monitoring wells, the availability of distinct isotope fractionation factors for the respective degradation pathways and biogeochemical conditions, and a detailed knowledge about the hydrology of the aquifer. Even if no specific isotope fractionation factor is available, a qualitative estimation of the biodegradation can be made by comparison with other isotope fractionation factors of known pathways and may provide valuable information on the processes likely to occur in the investigated aquifer. However, if multiple sources of contaminants with different initial isotopic signatures contribute to a mixed plume, this approach may be too complex for the reliable quantification of in situ biodegradation. In addition, the applicability of the approach is limited, if processes without significant isotope fractionation govern in situ biodegradation as shown for aerobic toluene degradation pathways catalyzed by mono- or dioxygenase enzymes reacting with the aromatic ring system [46].

The in situ microcosm approach (BACTRAP) with isotopic labeled contaminants and analysis of the isotope composition of lipid biomarkers provides an excellent tool for assessment by proving the microbial in situ activity in contaminated aquifers and for monitoring natural attenuation processes. This relatively simple, low-cost approach provides information in a reasonable time frame and the results are more reliable than ex-situ approaches. Even if the details of the metabolism of a target compound are not yet known, valuable information about the presumable pathway can be obtained by this approach. In particular, if metabolites of the parent compound can be identified on the bead material of the microcosms, a clear indication of the relevant degradation pathways can be given by this approach. Future investigations will provide the basis for a quantification of the biodegradation potential by this approach. Therefore, it is necessary to investigate the sorption behavior of the bead material in the presence and absence of bacteria. The key organisms related to contaminant degradation could not yet be identified; however, this aspect of the approach is currently under investigation. Future experiments to investigate the kinetics of the colonization as traced by quantitative PCR may elucidate the development of communities and may provide an indication for organisms supported by the test substrate. When the concentration of the label in the biomass is sufficiently high, stable isotope prob-

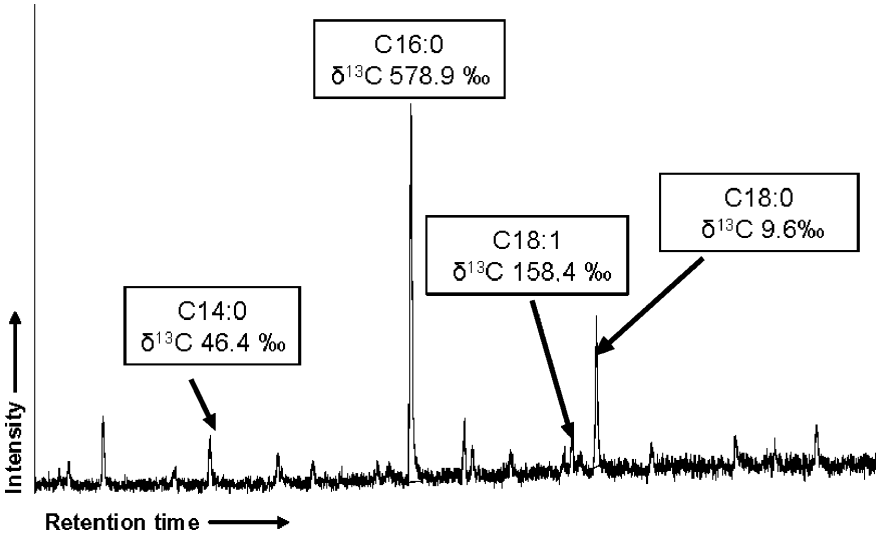

Figure 10. Total ion current of the fatty acid extracts from a BACTRAP amended with [$^{13}C_6$]-monochlorobenzene exposed for 49 d in an anaerobic aquifer in well Saf 4/97 at the Bitterfeld field site. Identified fatty acids and their isotope compositions are indicated in the boxes.

ing (SIP) can also be employed to identify organisms which use the ^{13}C-labeled contaminants as a carbon substrate [38, 40, 92]. This may open the "black box" of subsurface microbial ecology by linking structure and function.

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Anhang E

Sensitive Detection of Anaerobic Monochlorobenzene Degradation Using Stable Isotope Tracers (2007) und Supporting Information

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Sensitive Detection of Anaerobic Monochlorobenzene Degradation Using Stable Isotope Tracers

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Microbial degradation of monochlorobenzene (MCB) under anaerobic conditions was investigated using a stable isotope tracer under in and ex situ conditions. In situ microcosms were incubated directly in an anoxic aquifer and carbon derived from [$^{13}C_6$]-MCB was found to be incorporated into the microbial biomass. In laboratory microcosms, amended with [$^{13}C_6$]-MCB, anaerobic mineralization of MCB was indicated by the production of $^{13}CO_2$. Further, recovery of the ^{13}C-label in the fatty acids confirmed the assimilation of MCB-derived carbon into microbial biomass. The described approach may be applied to various other organic groundwater contaminants of concern using carbon (^{13}C) as well as other stable isotope tracers, such as nitrogen (^{15}N), allowing direct and sensitive detection of biodegradation.

Introduction

Monochlorobenzene (MCB) is a widespread groundwater contaminant found at many former chlorine chemistry sites particularly when chlorinated pesticides have been produced [www.who.int/en/]. The Bitterfeld/Wolfen region in Germany is a former production site for lindane (hexachlorocyclohexane) where today MCB is present as an ubiquitous contaminant throughout the entire aquifer with concentrations up to 30 mg L^{-1} (*1*−*3*). While MCB contamination may be the result of a spillage event, it can also be produced in situ by microbial lindane degradation or during dechlorination of higher chlorinated benzenes (*4*−*9*). MCB is the most mobile chlorobenzene due to its relatively high solubility and lower sorption potential to the aquifer matrix and usually long plumes form, indicating its high persistence in anaerobic aquifers. Drinking water limits for MCB in the United States are currently set at <0.1 mg L^{-1} [www.atsdr.cdc.gov/toxfaq]; in Germany, the quality target for surface waters is set at 1 μg L^{-1}, conform with European Union standards [www.umweltbundesamt.de]. While aerobic degradation of MCB has been well studied (for a review see van Agteren et al. (*6*)), complete anaerobic degradation and mineralization has, to our knowledge, not yet been reported. Only the complete anaerobic dehalogenation of the chlorinated benzenes to benzene has been described but the microbiota involved in the process have not been identified (*10*).

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In recent years, stable isotope approaches have been developed to investigate natural attenuation of contaminants. Stable isotope fractionation analysis may be used to investigate in situ biodegradation of pollutants (*11*); additionally, stable isotopes may be applied as tracer compounds (*12*−*15*). Previously, evidence for the biodegradation of MCB in an anoxic aquifer in Bitterfeld was provided by applying isotope fractionation techniques (*16*).

Recently, in situ microcosms (BACTRAPs), with application of stable isotope tracers, were developed and used to investigate degradation of BTEX compounds in the field (*12*, *14*, *15*). These in situ microcosms consist of a sorbent material serving as a surface for microbial growth and as a source for the isotopically labeled and unlabeled test substrates. During incubation of these microcosms in an aquifer for several months, microorganisms capable of degradation of the contaminant will incorporate carbon derived from the substrate into their biomass (e.g., fatty acids) and the biodegradation of the contaminant can be proven if the ^{13}C-label is recovered from the biomass. A preliminary investigation applying these in situ microcosms loaded with [$^{13}C_6$]-MCB supported the stable isotope fractionation analysis, providing evidence for the biodegradation of MCB under field conditions (*13*).

In this study, stable carbon (^{13}C) tracer methods were applied to investigate the biodegradation of MCB under anoxic conditions. In situ microcosm systems were incubated directly in the groundwater at the field site in parallel to a laboratory setup. Production of ^{13}C-labeled degradation products and incorporation of the ^{13}C into biomass were used to demonstrate anaerobic biodegradation of MCB.

Materials and Methods

Chemicals. The [$^{13}C_6$] chlorobenzene was purchased from Chemotrade Leipzig (Germany) with chemical and isotopic purity of >99%. The [$^{13}C_6$] chlorobenzene preparation further contained an impurity of 0.02% [$^{13}C_6$]-benzene. All other chemicals were obtained in p.a. quality or higher.

Site Description of the Aquifer in Bitterfeld (Saxony-Anhalt, Germany). The chlorobenzene (MCB)-contaminated aquifer is located near the city of Bitterfeld, Germany. Concentrations up to 30 mg L^{-1} of MCB and lower concentrations of chlorinated aliphatic compounds (up to 5 mg L^{-1}), benzene (up to 0.77 mg L^{-1}), and dichlorobenzenes (up to 3 mg L^{-1}) were found in the Quaternary aquifer (*16*). The time of spillage is not known but chlorobenzenes have been produced at the industrial site since the first decade of the last century. The hydrogeology and geochemistry of the aquifer system was described previously (*16*). Oxygen concentrations in the aquifer were analyzed using a flow-through chamber and an oxygen electrode (CellOx 325, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) and were generally below 0.3 mg L^{-1}, except for the highest level, close to the groundwater table where oxygen concentrations were around 1 mg L^{-1}. With use of this method, oxygen concentrations below 0.5 mg L^{-1} could not be resolved accurately. Significant nitrate concentrations were only found in the upper layers of the aquifer, indicating that nitrate reduction was not a relevant terminal electron acceptor (TEA) in the deeper layers of the investigated aquifer. Some iron and manganese reduction was indicated by low Fe(II) and Mn(II) concentrations. Elevated sulfide concentration associated with sulfate depletion indicated sulfate reduction and sulfate concentrations up to 1.48 g L^{-1} suggested that sulfate was available as the principle TEA in this aquifer (*16*). Elevated methane concentrations were not found in the

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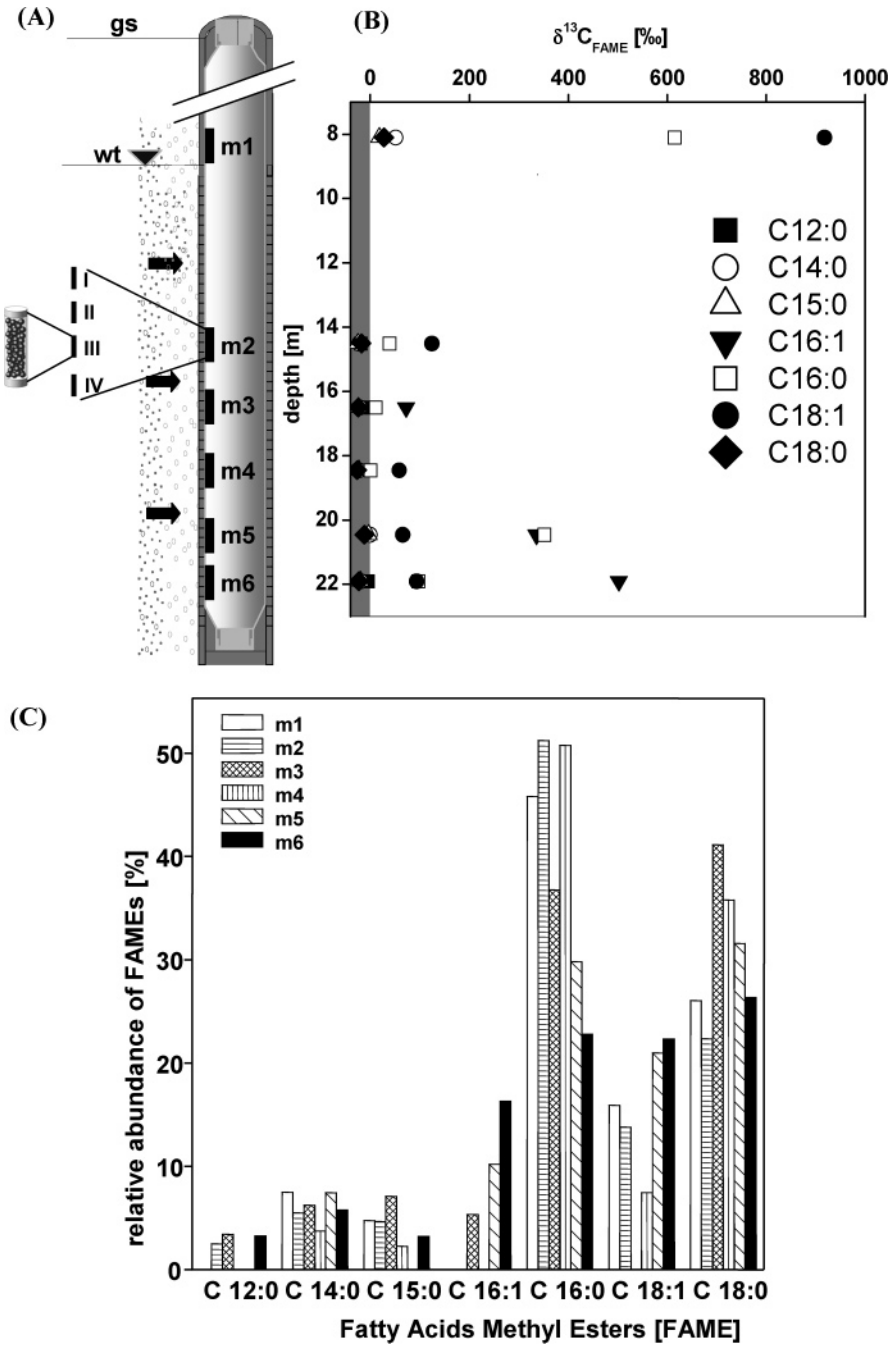

FIGURE 1. (A) Multilevel sampling system (MLPS), preventing groundwater circulation and penetration of oxygen from the surface, used for the in situ microcosm experiment in well SAF4. The black rectangles indicate the location of the in situ microcosms (m1−m6) in general between the filter screen of the well and the membrane of the MPLS, whereas the small rectangles (I−IV) show the detailed experimental setup with [^{12}C]-MCB (I), Blank (II), [$^{13}C_6$]-MCB (III), and Blank (IV) in situ microcosms as it was performed in every depth. The black arrows show the groundwater flow direction: gs, ground surface; wt, water table. (B) Carbon isotope composition of fatty acid methyl esters (FAME) extracted from [$^{13}C_6$]-MCB amended in situ microcosms incubated for 7 weeks at different depths (m1−m6) at the multilevel monitoring well SAF4 in Bitterfeld. The gray shaded area indicates the natural abundance of $\delta^{13}C_{FAME}$. (C) Relative abundance of total lipid fatty acid methyl ester extracted from in situ microcosm loaded with $^{13}C_6$-labeled MCB incubated in different depths (m1−m6) at the monitoring well SAF4 in Bitterfeld.

aquifer so methanogenesis apparently was not an important process. Overall, the geochemical analysis points at prevalently anoxic conditions in the aquifer.

Preparation and Incubation of in Situ Microcosms. The in situ microcosms were prepared as described before by Stelzer et al. (*15*). Briefly, Bio-Sep beads (University of Tulsa, Tulsa, OK) were loaded with MCB as carbon substrate for microorganisms via gas phase under reduced pressure. The in situ microcosms for the experiments in Bitterfeld were amended with [$^{13}C_6$]-MCB or natural abundance MCB to a concentration of 76 mg g^{-1} sorbent material. In parallel, unamended in situ microcosms (blank) were deployed. Material from these control experiments was used to investigate the isotope composition of fatty acids at natural abundance. The in situ microcosms were incubated for 7 weeks in groundwater well SAF4 equipped with a multilevel sampling device (MLPS) to avoid any oxygen penetration and groundwater circulation between depths (see Figure 1a; Figure S1 Supporting Information) (*17*). In the MLPS experiments the microcosms were placed in stainless steel cages

as a spacer to ensure a permanent flow of groundwater through the in situ microcosms and to avoid contamination with biological material by direct contact with the packer membrane or the filter screen of the well. The set of in situ microcosms used as an inoculum for the enrichment cultures were incubated directly in the groundwater in the wells without a MLPS or stainless steal cages.

Extraction and Derivatization of Fatty Acids (FA). After removal of the in situ microcosms from the groundwater well, the Bio-Sep beads were extracted using a dichloromethane−methanol−water mixture as solvent modified after Blight et al. (*18*). For investigation of the laboratory microcosms and enrichment cultures, 15 or 3 mL of liquid, respectively, was removed from the microcosms for extraction with the dichloromethane−methanol mixture. After phase separation the dichloromethane phase containing the fatty acids (FA) was evaporated to dryness and derivatized using a trimethylchlorosilane (TMCS)−methanol mixture (1:8; v:v) as reactant for 2 h at 70 °C to obtain total lipid fatty acid methyl esters (FAME) (*19*). After evaporation to complete dryness, the FAME fraction was dissolved in *n*-hexane for subsequent analysis using a gas chromatograph coupled to a mass spectrometer (GC-MS) and a gas chromatography-combustion-isotope-ratio-monitoring-mass-spectrometer system (GC-C-IRMS) (see Supporting Information). FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco, Munich, Germany) and concentrations of FA were quantified relative to the internal standard (henicosanoic acid, C21:0). The fatty acids are designated in the form of *A:B* where A is the number of carbon atoms and B is the number of double bonds.

Laboratory Microcosms. (1) Microcosms. Groundwater was taken from two wells (SAF11 and SAF30) at the MCB contaminated field site in Bitterfeld. The filter screens of the wells are at 19 to 20 and 19 to 21 meters below ground, respectively. After transport to the laboratory and transfer into an anaerobic glovebox (98−96% N_2/2−4% H_2), the groundwater was amended with 1 mg L^{-1} of resazurin as a redox indicator (*20*). The microcosms were prepared in 120 mL vials to which 100 mL of the groundwater mixture was added. The vials were closed with Teflon-coated gray butyl rubber stoppers and crimped with aluminum crimps. For each well, 11 microcosms were prepared. Of each set, two microcosms were autoclaved for 40 min on 3 consecutive days to prepare killed controls. Then, 5 of the live microcosms and the 2 killed microcosms were amended with natural abundance MCB and the other 4 with [$^{13}C_6$]-MCB to a concentration of approximately 100 μmol L^{-1}. MCB concentrations were followed by GC-FID analysis. CO_2 concentrations were estimated by GC-C-IRMS vs an external calibration curve. SAF11 and SAF30 contained 6.7 and 3.7 mmol L^{-1} CO_2, respectively. For details of the analysis see Supporting Information. Sulfide concentrations were determined according to the method of Cline (*21*). Iron(II) concentrations were determined by the Department of Analytics at the UFZ-Centre for Environmental Research using the Spectroquant Iron test (Merck, Darmstadt, Germany).

(2) In Situ Microcosm Enrichment. In situ microcosms loaded with natural abundance MCB from a preliminary experiment incubated for approximately 8 weeks in the groundwater wells SAF11 and SAF30 were used as an inoculum for the enrichment cultures. Therefore, the in situ microcosms were recovered after incubation and the sorbent material was added to 27 mL anaerobic culturing tubes filled with groundwater from the monitoring wells. The tubes were closed with Teflon-coated gray butyl rubber stoppers and crimped. In the laboratory, the tubes were amended as follows with anoxic sterile stock solutions to a final concentration of 1 mg L^{-1} resazurin, 2 mg L^{-1} yeast extract, 2 mg L^{-1} vitamin B_{12}, 1 mM Na_2S, and either for the sulfidogenic enrichments 5 mM $FeSO_4$ or for the methanogenic enrichments 2 mM $FeCl_2$. Approximately 20 μmol of MCB was added to each tube. Due to sorption of MCB to the Bio-Sep beads, it was not possible to quantify MCB in these enrichment cultures. Qualitatively, MCB appeared to decrease over time and, apparently, microbial conversion occurred (data not shown). After incubation of approx 4 month, subcultures were made from each in situ microcosm enrichment. Liquid from each microcosm was diluted (10%) into fresh basal medium according to the description by Zinder (*22*) amended with 0.1% $NaHCO_3$, vitamin solution, 1 mM Na_2S, and as indicated 5 mM $FeSO_4$ or 2 mM $FeCl_2$. Two parallels were prepared for each transfer set: one culture was amended with natural abundance MCB while the second culture was amended with [$^{13}C_6$]-MCB to a concentration of 500 μmol L^{-1}.

TABLE 1. Carbon Isotope Composition of Benzene and MCB Extracted from Blank, Unloaded, and [$^{13}C_6$]-MCB Loaded in Situ Microcosms Incubated for 7 Weeks at Different Depths at the Multilevel Monitoring Well SAF4 in Bitterfeld[a]

depth (m)	blank $\delta^{13}C_{Benzene}$ [‰]	blank $\delta^{13}C_{MCB}$ [‰]	labeled $\delta^{13}C_{benzene}$ [‰]
m1 (8.1)	3590	190	n.a.
m2 (14.5)	1000	265	1310
m3 (16.5)	485	245	3940
m4 (18.5)	1080	50	n.a.
m5 (20.5)	700	60	8520
m6 (21.9)	560	−25.1	3780

[a] n.a. = not analyzed. The standard deviation for the analysis of the carbon stable isotope composition was <1‰ with $\delta^{13}C$ < 500‰ and between 5 and 10‰ with $\delta^{13}C$ > 500‰.

Results and Discussion

In Situ Microcosms. The recently developed in situ microcosms were directly incubated in the anoxic aquifer in Bitterfeld to investigate the MCB degradation (*12*, *14*, *15*). Previous studies applying isotope fractionation techniques and a preliminary in situ microcosms study provided first evidence that MCB was degraded within this aquifer (*13*, *16*). The in situ microcosms were installed inside a monitoring well in a multilevel fashion (microcosm set m1−m6; see Figure 1a). The use of a multilevel sampling device allowed depth discreet sampling and should prevent circulation of groundwater between the different levels and entry of oxygen to the microcosms during exposure (*17*). However, water may circulate between different levels through the filter material of the well screens or the surrounding sediments. After incubation in the groundwater for 7 weeks, the in situ microcosms were removed from the monitoring well and used for further analysis. Direct extraction of the sorbent material from the in situ microcosm showed that, in addition to the parent [$^{13}C_6$]-MCB, ^{13}C-labeled benzene was present at several of the investigated depths (m2, m3, m5, and m6) with an isotope signature of $\delta^{13}C$ > 1000‰ (see Table 1). ^{13}C-labeled benzene was also observed in blank, nonamended microcosms. At depth m6, ^{13}C-labeled benzene ($\delta^{13}C$ = 560‰) and MCB with an isotope composition typical for this aquifer section ($\delta^{13}C$ = −25 ‰) was observed in the blank, nonamended microcosm. Both compounds must have been adsorbed during incubation on the nonamended microcosm from the surrounding groundwater and were present in concentrations in a similar order of magnitude; benzene concentrations were approximately one-third that of MCB (see Figure S2, Supporting Information). Although [$^{13}C_6$]-benzene was already present in trace concentrations (0.02%) in the commercial [$^{13}C_6$]-MCB, it is unlikely that the labeled benzene was derived from the impurity of the chemical. Since benzene and MCB have similar physicochemical properties,

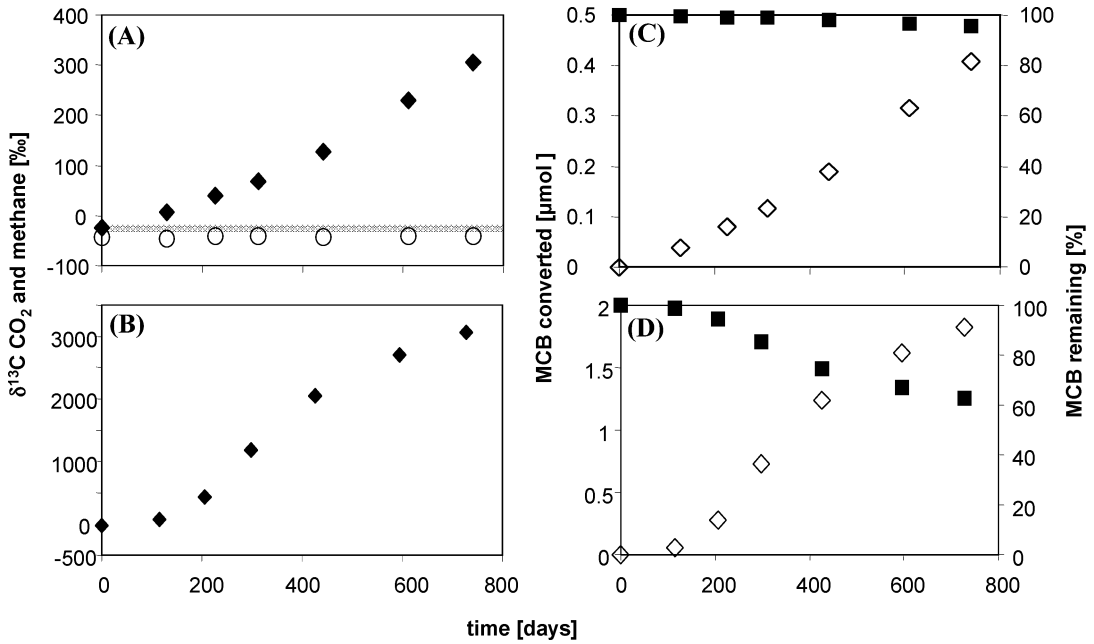

FIGURE 2. Carbon isotope signature of CO_2 (◆) and methane (○) in (A) a microcosm prepared with groundwater from SAF11 and (B) the enrichment culture. Based on the CO_2 content and isotope signature, the amount of [$^{13}C_6$]-MCB degraded was calculated in micromoles per microcosm (◇) and % remaining of the total added [$^{13}C_6$]-MCB (■) for (C) the microcosm and (D) the enrichment culture. The shaded area in panel A indicates the observed isotope signature of CO_2 in the control microcosms.

it would not be probable that a separation of both chemicals would occur upon diffusion or transport with the groundwater, resulting in sorption of labeled benzene without the co-transport of labeled MCB. The results suggest that benzene is formed upon dehalogenation of MCB in the aquifer. Additionally, at the field site, benzene could be detected in the aquifer in low concentrations (up to 20 μg L^{-1}).

To investigate assimilation of MCB-derived carbon into bacterial biomass, the isotope composition of fatty acids (FA) was determined. FA extracted from the in situ microcosm systems loaded with [$^{13}C_6$]-MCB were significantly labeled, providing evidence for the complete degradation of MCB via central metabolism and assimilation of MCB-derived carbon for synthesis of biomass (Figure 1b). The relative abundance of the various identified FA was similar, comparing the microcosms incubated at the different depths m1 to m6 (Figure 1b). C16:0 and C18:0 were the most abundant FA, followed by lower amounts of the mono-unsaturated C16:1 and C18:1. Only minor amounts of C12:0, C14:0, and C15:0 were detected (Figure 1c). Close to the groundwater table (m1), all identified FA were labeled. Possibly, at the transition to the capillary fringe oxygen (1 mg L^{-1} at m1) is available as an electron acceptor for degradation in this zone (*16*); therefore, MCB may be degraded aerobically at the upper level. At deeper levels (m2−m6), conditions were strictly anaerobic and significant enrichment in ^{13}C was observed in the hexadecanoic acid (C16:0; up to $\delta^{13}C = 352‰$ at depth m6), hexadecenoic acid (C16:1; up to $\delta^{13}C = 503‰$ at depth m5), and the octadecenoic acid (C18:1; $\delta^{13}C = 94‰$ at depth m6) (Figure 1a,b). Other FA were not labeled significantly. The incorporation of ^{13}C derived from [$^{13}C_6$]-MCB into FA provided clear evidence for in situ biodegradation of MCB under the environmental conditions present in the aquifer.

Laboratory Microcosms and Enrichments. Laboratory microcosms were used in parallel to investigate anaerobic MCB degradation in a closed system and to attempt cultivation of MCB degrading microorganisms. A first set of laboratory microcosms was prepared from a mixture of groundwater derived from the same MCB plume as described above. A decrease in MCB concentration was observed in several microcosms amended with electron donors (e.g., lactate, benzoate) or acceptors (sulfate), but no intermediates or degradation products were found and the activity could not be sustained (data not shown). For this reason in a second setup of microcosm systems, we supplied isotopically labeled MCB to enable a more sensitive detection of the degradation processes. Two separate sets of microcosms were prepared with groundwater from wells SAF11 and SAF30 and ^{13}C-ring labeled MCB was used as a tracer substance. No MCB decrease relative to the killed control could be observed (data not shown). The concentrations and isotope signatures of benzene, methane, and CO_2 were monitored over time. In Figure 2a an example is given of the observed isotope signatures for methane and CO_2 in the investigated time frame. The concentrations of CO_2 did not change significantly over the investigated period (data not shown), but the CO_2 became clearly enriched in ^{13}C (Figure 2a). Over time, the isotope signature of CO_2 increased from $\delta = -24.4$ to +305‰ in the microcosms prepared from groundwater of well SAF11 (Figure 2A). The isotope signature of CO_2 in the microcosms prepared with groundwater of well SAF30 changed similarly ($\delta = -25.3$ to +443‰) (data not shown). All replicates of microcosms in both sets (SAF11, SAF30) showed enrichment of the ^{13}C-label in the CO_2 fraction, although the lag period for the degradation was highly variable (data not shown). After 700 days of incubation, all microcosms amended with [$^{13}C_6$]-MCB had significantly produced $^{13}CO_2$. Production of $^{13}CO_2$ was not observed in any of the killed controls amended with [$^{13}C_6$]-MCB. The enrichment of ^{13}C in CO_2 clearly showed the mineralization of MCB in these microcosms. Additionally, the isotope signature of methane remained stable around $\delta = -40$ to −45‰ and $\delta = -45$ to −50‰ for microcosms from wells SAF11 and SAF30, respectively, and no methane production was observed. Apparently, methanogenesis was not an important process involved in MCB degradation in our laboratory microcosms. Resazurin was added to the microcosms, allowing visual monitoring of anoxic conditions during the incubation time. All microcosms remained clear throughout the incubation period, indicating a reduction potential of < -0.051 mV (*20*). At the end of the study period, soluble Fe(II) and total sulfide was measured to confirm the presence of anoxic conditions and redox buffers in the system

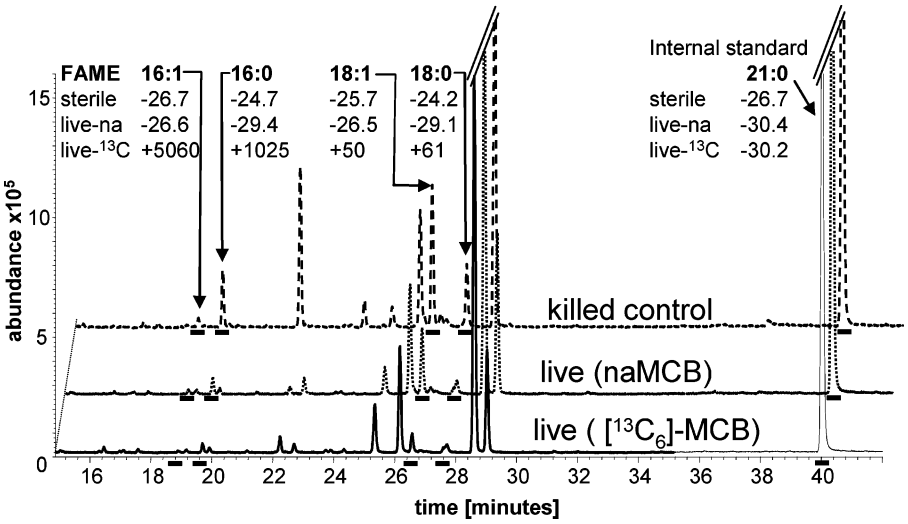

FIGURE 3. Total ion current of fatty acid methyl esters derived from microcosms prepared with aquifer water of well SAF30. Shown are samples from a killed control, natural abundance MCB amended, and [$^{13}C_6$]-MCB amended microcosms. Identified peaks are labeled and underlined (_). FAME were identified via GC-MS and the isotope signature in the various samples are shown in δ‰ [VPDB].

(see Table S1, Supporting Information). Sulfide concentrations were in the same order of magnitude, generally a bit higher, compared to the original groundwater while soluble Fe(II) concentrations were lower, confirming anoxic conditions. It should be considered that Fe(II) concentrations could have been underestimated due to precipitation with sulfide. In these microcosms, early in the incubation period (100−300 days), low levels of ^{13}C-labeled benzene could be identified by the means of retention time using headspace analysis, potentially derived from the impurity in the labeled MCB preparation. Benzene did not accumulate and disappeared over time, suggesting its conversion in the microcosms.

With use of the concentration of CO_2 (6.7 or 3.7 mmol L^{-1} for microcosms from SAF11 or SAF30, respectively) and corresponding isotope signatures in the ex situ microcosms, we estimated the amount of MCB mineralized as shown in Figure 2C for the microcosms from SAF11. After 700 days of incubation, 3 out of 4 microcosms from each set incubated with the labeled MCB were estimated to have converted 3 to 4% of the added MCB. Interestingly, in each set, one microcosm had converted up to 11 and 30% of MCB based on $^{13}CO_2$ evolution for microcosms derived from groundwater from wells SAF30 and SAF11, respectively.

With use of the analysis of the stable isotope signature of CO_2, degradation could be determined extremely sensitively. A significant enrichment in ^{13}C (e.g., $\delta = +50$‰) would, in these microcosms from SAF11, represent an estimated degradation of about 100 nmol of MCB, corresponding to about 1% of the total added MCB. This order of sensitivity to measure biodegradation would be impossible to obtain using other methods except for methods using radioactive carbon tracers.

To prove incorporation of MCB-derived carbon into microbial biomass, fatty acids were extracted from the laboratory microcosms and derivatized and FA isotope signatures were analyzed. In Figure 3 the total ion currents of three selected samples from the set SAF30 are shown. While the amount of biomass was highly similar in the different samples, [$^{13}C_6$]-MCB amended microcosms showed FA that were significantly enriched in ^{13}C (16:1, 16:0, 18:0, and 18:1 with $\delta = +5060$, +1025, +50, and +61‰, respectively), indicating incorporation of MCB-derived carbon into biomass. In a comparison of the three samples, a shift in FA composition could be observed as octadecanoic acid (18:0) was present in the killed control microcosm but nearly absent in the live microcosms. Since the fatty acids found are common for most bacteria and have relatively low specific taxonomic value, no conclusions can be drawn about the microbial community involved in the biodegradation (*23*).

According to the literature, no anaerobic MCB mineralizing microbial cultures have been described yet. In an attempt to improve the cultivability of anaerobic MCB degrading microorganisms, the sorbent from in situ microcosms was used as an inoculum. Therefore, after preenrichment of organisms during approximately 8 weeks exposure in wells SAF11 or SAF30, the in situ microcosms were removed and the sorbent material was used as an inoculum for enrichments. In these initial enrichments, disappearance of MCB was observed, but quantification of MCB was impossible due to the adsorption of MCB on the sorbent material, resulting in only very low dissolved concentrations in the enrichment cultures. After approximately 4 months, 10% liquid transfers were prepared from these enrichments into a fresh medium and [$^{13}C_6$]-MCB was added as a tracer. Methane, CO_2, and benzene concentrations as well as their isotope compositions were monitored in these transfer cultures (see Figure 2c). The appearance of $^{13}CO_2$ was observed but, again, no labeled methane was formed. An analysis of the incorporation of carbon into biomass was also attempted. FA were analyzed but concentrations were too low for a quantitative assessment. On the GC-C-IRMS, labeled products could be observed in the isotope trace at the same retention time as commonly found FA (C16:1, C16:0, C18:1, and C18:0), implying the presence of labeled biomass (data not shown). Apparently, only small quantities of biomass were formed in our enrichment cultures, which corresponded with the absence of obvious biomass formation and turbidity.

Two major hypothetical degradation pathways would come into consideration for the anaerobic biodegradation MCB: (1) initial dechlorination, followed by the degradation of benzene or (2) direct attack on the benzyl ring with the formation of a substituted chlorinated aromatic substance (e.g., chlorobenzoate and chlorophenol). The first pathway would suggest a similar pattern as observed previously by Nowak (*10*). Benzene could then be further degraded via benzoate or phenol as intermediates (*24*−*28*). Alternatively, a chlorinated phenol, benzoate, or toluene could be formed which then is dechlorinated via reductive dechlorination as described for chlorinated benzoate to benzoate by, e.g., *Desulfomonile tiedje* or for chlorinated phenols to phenol by

Desulfitobacterium spp (*29*−*31*). Although the observed traces of benzene on blank in situ microcosms suggest degradation via dehalogenation, because of the impurity of benzene in the labeled MCB preparation the first pathway could not be verified in the laboratory experiments. However, the in situ experiments indicated that reductive dehalogenation may occur in the aquifer. Further detailed investigations of the pathway and development of a stable mixed culture and further isolation of microorganisms involved in the anaerobic MCB degradation are required.

To summarize, for the first time we could demonstrate the sensitive detection of anaerobic biodegradation of MCB, which was thus far considered persistent, in situ and in ex situ laboratory studies. A previous investigation supported MCB degradation in an anaerobic aquifer via stable isotope fractionation analysis but no definite proof for anaerobic degradation was presented thus far (*16*). By means of stable isotope tracers (^{13}C), we provided evidence for anaerobic MCB degradation, although only very small portions of the contaminant (nmol) were mineralized. Our concept based on the use of ^{13}C labeled tracers in combination with in situ and laboratory microcosms was found very valuable for analysis of the slow degradation of recalcitrant organic chemicals. The application of modern analytical techniques for isotope analysis enables stable isotope probing of cell components (FA), allowing the sensitive elucidation of carbon fluxes on a molecular level. Although stable isotope probing of DNA or RNA could give valuable information about the microbial community involved in the degradation of MCB, application of these techniques in our field and laboratory systems is currently not possible yet due to the lack of biomass formed.

The described approach can be applied to detect the biodegradation and to investigate the persistence of a variety of recalcitrant environmental pollutants using ^{13}C or ^{15}N stable isotope tracers which are needed for the synthesis of cell components. Furthermore, we can use the in situ microcosms as pre-enrichments to improve cultivation efficiency of the microbial community involved in the degradation processes. In the future, our concept may be applied to investigate the environmental fate of recalcitrant organic chemicals, providing valuable support for evaluation of chemicals for regulatory framework such as Registration, Evaluation and Authorisation of Chemicals (REACH) recently developed in Europe.

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Supporting Information Available

Additional information is available on the detailed methods for analysis using GC-MS and GC-C-IRMS, the analysis of the in situ and laboratory microcosms.

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SUPPORTING INFORMATION

Sensitive detection of anaerobic monochlorobenzene degradation using stable isotope tracers

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METHODS

Analysis

GC-MS. For identification and structural characterization of FAME by GC-MS a Hewlett Packard 6890 gas chromatograph coupled to a 5973 quadropole mass spectrometer (Agilent, Palo Alto, USA) was used. The carboxylic acid fraction was separated on a BPX-5 column (30 m*0.32 mm*0.25 µm) (SGE, Darmstadt, Germany) with a temperature program of 120°C initial temperature for 4 min, heat at 4°C min^{-1} to 250°C, heat at 20°C min^{-1} to 300°C, and hold for 10 min. FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco) and concentrations of FA were quantified relatively to the internal standard (Henicosanoic acid, C21:0). The fatty acids are designated in the form of *A:B* where A is the number of carbon atoms, B is the number of double bonds.

GC-FID For detection of MCB, ethene and the chlorinated ethenes in the microcosms and enrichment cultures, gas chromatography with flame ionization detection (GC-FID) (gas chromatograph:Varian Chrompack CP-3800, Middelburg, The

Netherlands), equipped with a 30m x 0.53mm GS-Q column (J&W Scientific, Waldbronn, Germany), was applied. The temperature program used was as follows: 1 min at 100^{o}C, 50^{o}C min^{-1} to 225^{o}C, hold 2.5 min. The FID was operated at 250^{o}C and helium was used as carrier gas (10 psi). The injection was automated using an HP 7694 headspace auto sampler (Hewlett Packard, Palo Alto, USA), adding 0.5 ml headspace samples to 10 ml auto sampler vials flushed with helium, which were closed with a Teflon coated butyl rubber septum and crimped.

GC-C-IRMS. The carbon isotope composition of the carboxylic acids fractions was analyzed using a gas chromatography-combustion-isotope-ratio-monitoring-mass-spectrometer system (GC-C-IRMS). The system consists of a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany). The performance was described previously (1,2). A BPX-5 column (50 m*0.32 mm*0.5) (SGE, Darmstadt, Germany) was used for chromatographic separation with helium as carrier gas at a flow rate of 1.5 mL min^{-1} and a temperature program with initial temperature of 60°C for 2 min, heat at 20 °C min^{-1} to 120 °C, heat at 2 °C min^{-1} to 300 °C, and hold for 20 min (3). The analysis of chlorobenzene on the same system was described previously (4). For separation of methane and CO_2 the same system was used equipped with a Poraplot Q column (25 m x 0.32 mm ID, 1 µm film; Chrompack, Middelburg, The Netherlands). For the separation on a Poraplot column, the temperature was set to 40°C isothermally. 50-100 µl headspace samples were injected at 1:5 to 1:50 split ratio. The carbon isotope ratio of fatty acids is reported in δ-notation (per mill) relative to the Vienna Pee Dee Belemnite standard (V-PDB) with known isotopic composition (5).

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Investigation of in situ microcosms

The *in situ* microcosms were incubated in a multilevel fashion, attached along the complete length of the multi-level sampling system (MLPS) at 6 depths (m1-m6) at 8.1, 14.5, 16.5, 18.5, 20.5 and 21.9 m depth below the surface. The water table was located at approx. 4 meters below the surface. The *in situ* microcosm sets, consisting of a ^{12}C-MCB amended microcosm, an unloaded microcosm (blank), a ^{13}C-MCB amended microcosm and a further blank were positioned over a depth of about 80 cm. The space between the individual *in situ* microcosms of each level was approx. 20 cm (See Fig. S1). According to previous experience, this distance is sufficient to allow the packer membrane to separate the microcosms within the well and prevent circulation of water between the individual microcosms. However, water may circulate between different levels through the filter material of the well screens or the surrounding sediments.

During incubation, the Bio-Sep® bead material in the unloaded microcosms can adsorb contaminants from the surrounding ground water and aquifer microorganisms may grow on the surfaces using the adsorbed contaminants as carbon source. The isotope composition of contaminants adsorbed from the groundwater to the blank microcosm is commonly identical to the isotope composition of individual contaminants in the aquifer. On one of the blank experiments at the level m6 the isotope signature of MCB was comparable to the surrounding groundwater (-25 ‰ ± 0.5), however, benzene was significantly enriched in ^{13}C (+560 ‰) (Fig. S2). The label in benzene can only stem from one of the ^{13}C-substrate amended microcosms. Because the packer system should have prevented direct circulation in the open tube of the well, the ^{13}C-labeled material must have been transported through the filter section of the well or the surrounding sediments from the ^{13}C amended microcosm to

the blank over a distance of at least 20 cm (See Fig. S1). The relative amounts of MCB and benzene adsorbed during incubation on the non amended microcosm were similar with the relative benzene concentrations on the blank *in situ* microcosm being about one third of the MCB concentration. (See Fig. S2). Although [$^{13}C_6$]-benzene was already present in trace concentrations (0.02 %) in the commercial [$^{13}C_6$]-MCB, it is unlikely that the impurity of the chemical was the source of labeled benzene on the blank microcosm. Because benzene and MCB have similar physico-chemical properties, both compounds should be transported in the same way and a separation of both chemicals upon diffusion in or transport with the groundwater is very unlikely. Therefore, transport of labeled benzene without the co-transport of labeled MCB seems to be unlikely and the adsorption of small parts of the substrate ([$^{13}C_6$]-MCB with 0.02% ^{13}C-benzene) and the mixture of MCB and benzene from the aquifer contamination would lead to a very different isotope signatures. The GC-C-IRMS analysis showed that the concentrations of benzene and MCB were in a similar range but only benzene contained the ^{13}C label (Figure S2). Therefore it is unlikely that the labeled benzene was derived from the impurity in the substrate. It is likely that the ^{13}C-labeled benzene was formed via dehalogenation during transport from the ^{13}C-amended microcosms to the blank. The relatively high concentration of MCB and benzene on the blank may further indicate that benzene may be formed to some extent during reductive dehalogenation in this part of the aquifer. It should be mentioned that we also found ^{13}C-labeled benzene in the laboratory microcosms but because of the impurity in the ^{13}C-MCB preparation we could not prove its production. However, benzene did not accumulate in these laboratory microcosms and disappeared over time.

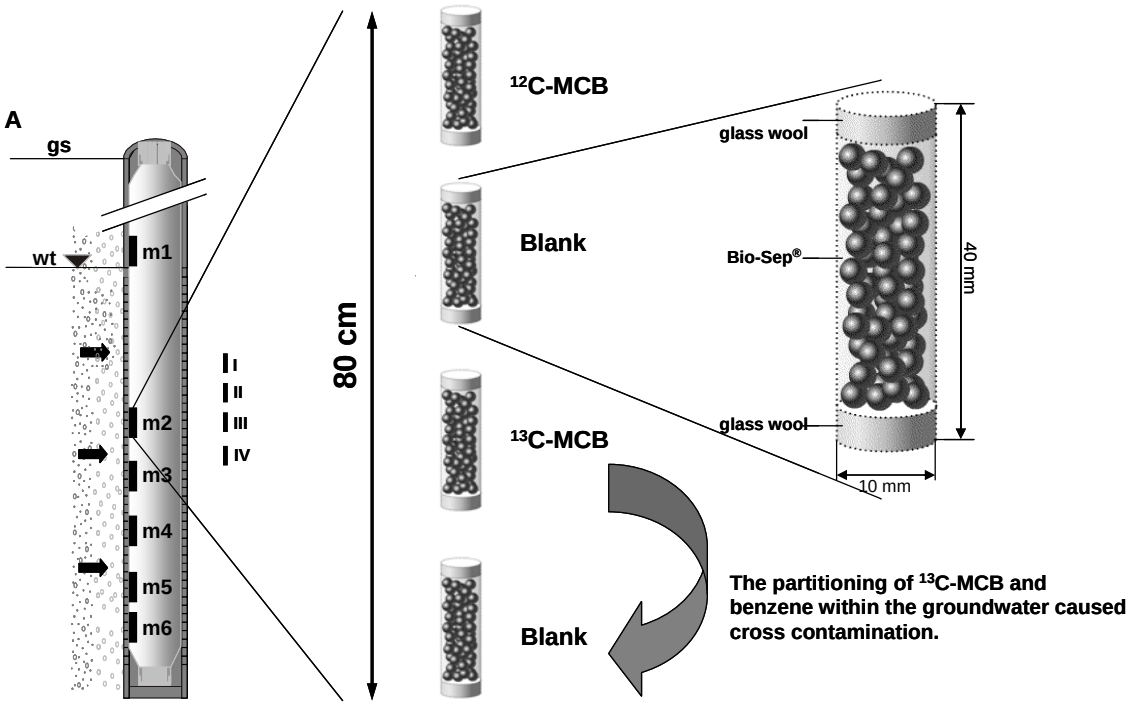

Figure S1:

Detailed lay-out of the *in situ* microcosm experiment. The *in situ* microcosms were incubated at 6 levels (m1-m6) and each set consisted of a ^{12}C-MCB loaded, a blank, a [$^{13}C_6$]-MCB-loaded and another blank microcosm. The overall distance of each set was about 80 cm, each microcosm was located approx. 20 cm from the next.

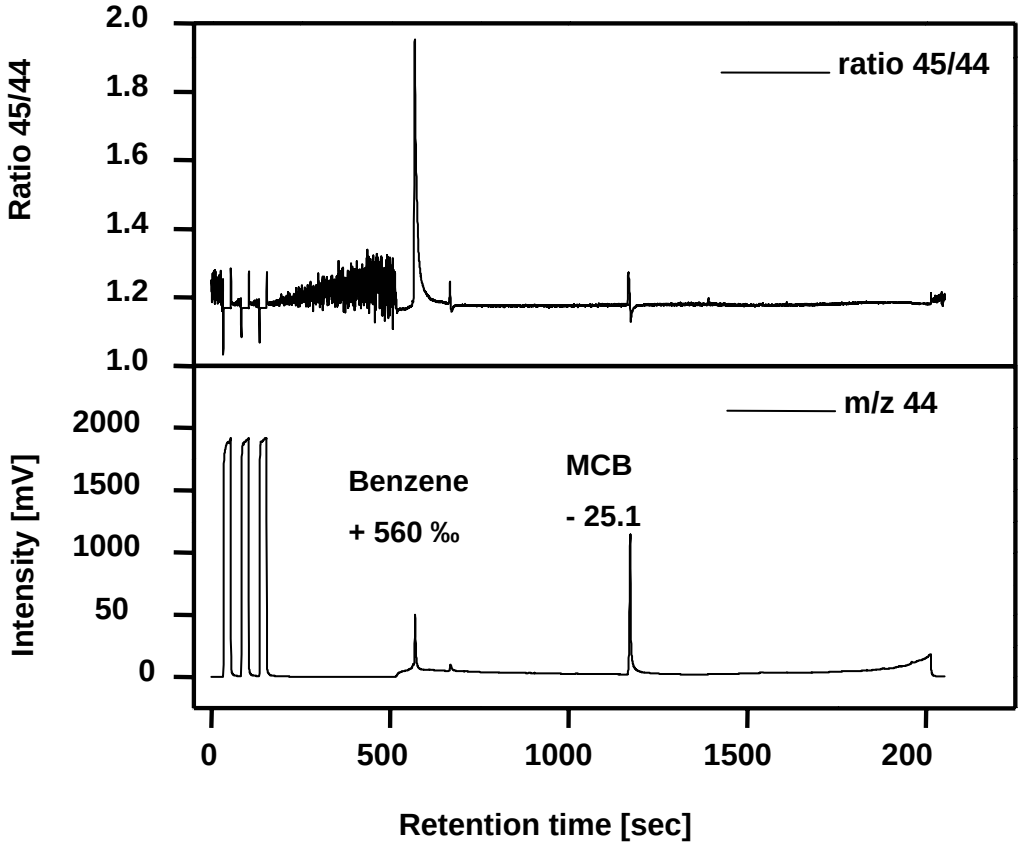

Figure S2:

GC-C-IRMS-chromatogram of the dichloromethane extract from a Blank (non-loaded) *in situ* microcosm (m6) incubated at SAF4. **A:** Only the benzene shows an enrichment in ^{13}C indicated by the increase in the ratio of 45/44 whereas the MCB shows an isotope signature within the range of natural abundance. **B:** The relative concentration of both compounds is displayed (m/z 44= $^{12}CO_2$. In addition the $\delta^{13}C$ values of MCB and benzene are indicated in the lower panel.

Confirmation of anoxic conditions in the laboratory microcosms:

Resazurin was used as a redox indicator to monitor anaerobic conditions throughout the microcosm experiment. At -0.051 mV and below at neutral pH, resazurin will be colorless indicating anaerobic conditions while at higher reduction potential it will turn to pink color (Tratnyek et al, 2001). Visual inspection of the microcosms on a regular basis indicated the presence of anoxic conditions. The presence of redox active compounds (Fe(II) and S^{2-}) (Table S1) which would be abiotically oxidized in the presence of oxygen, support the presence of anoxic conditions in the microcosms. Sulfide concentrations are in the same range, or sometimes higher, in the microcosms after 800 days compared to the original groundwater. Dissolved Fe(II) concentrations were generally found to be lower compared to the groundwater, but the total Fe(II) concentrations may be underestimated since FeS precipitates may form. Both the visual inspection using resazurin and the presence of Fe(II) and sulfide in the microcosms support the presence of anoxic conditions in the microcosms throughout the experiment.

Reference:

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Table S1:

Soluble iron (II) and total sulfide concentrations were determined to confirm the presence of anaerobic conditions and redox buffers in our microcosms. Shown are values for the original groundwater from well SAF11 and in the anaerobic microcosms prepared from this groundwater at the end of the experiment (approx. 800 days). *Values for the microcosm depicted in Figure 2 of the main manuscript. **Data from Kaschl, A.; Vogt, C.; Uhlig, S.; Nijenhuis, I.; Weiss, H.; Kästner, M.; Richnow, H. H. Isotopic fractionation indicates anaerobic monochlorobenzene biodegradation Environ Toxicol Chem 2005, 24, 1315-1324.

Sample	Fe (II) µmol L^{-1}	S^{2-} µmol L^{-1}
SAF11 groundwater	21**	22**
SAF11 [labeled] microcosms live	3.8*	26.3*
	3.6	17.5
	4.2	33.3
	3.9	38.5
SAF11 [unlabeled] microcosms live	4.1	0
	3.8	47.4
	3.6	56
	4.5	36.8
	4.2	38.5
SAF11 microcosms killed control	8.2	0
	6.1	0

Anhang F

Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers (2008) und Supporting Information

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Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers

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KEYWORDS

Monochlorobenzene (MCB), Dichlorobenzene (DCB), Compound-Specific Stable Isotope Analysis (CSIA), Anaerobic Degradation, Principal Component Analysis (PCA), Natural Attenuation (NA)

ABSTRACT

Assessment of intrinsic microbial activity under anoxic conditions at a chlorobenzene contaminated field site was addressed by hydrogeochemical analysis, stable isotope tools and multivariate statistics to elucidate the fate of chlorinated benzenes with particular emphasize on most persistent monochlorobenzene (MCB). In situ microcosm analysis provided evidence of microbial assimilation of MCB-derived carbon and laboratory investigations asserted mineralization of MCB at very low degradation rates. To further investigate in situ biodegradation of chlorinated benzenes (CBs), compound-specific stable isotope analysis (CSIA) was applied. During sequential degradation of CBs the isotope signature of a particular chlorobenzene species might be affected simultaneously by depletion and enrichment of ^{13}C, complicating the application of the Rayleigh approach for quantification of in situ degradation. Therefore, an isotope balance concept was applied. The enrichment of the cumulative isotope composition of all CBs indicated in situ biodegradation. Additionally, the linkage between geochemistry, contaminant concentration and the observed degradation activity was investigated by principal component analysis (PCA) underlining hydrogeochemical heterogeneity at the study site. The application of an integrative approach relying on multiple lines of evidence to document that capability of the intrinsic microbial community to anaerobically degrade MCB and DCB was found useful to characterize the natural attenuation potential of the site.

SYNOPSIS

Multiple lines of evidence demonstrated in situ degradation of chlorinated benzenes in an anoxic aquifer.

INTRODUCTION

Chlorinated aromatic compounds are worldwide intensively used to synthesize pesticides and other chemicals leading to ubiquitous distribution in the environment (1). Chlorinated benzenes (CBs) can also be formed during anaerobic microbial transformation of hexachlorocyclohexane, of which the γ-isomer (Lindane) is worldwide used as pesticide (2,3). Due to their toxicity, persistence and accumulation in the food chain CBs are of great environmental concern.

CBs may be subjected to both aerobic and anaerobic microbial degradation (1,3,4). With increasing chlorination CBs may undergo reductive dehalogenation (RDH) either as cometabolic reaction or energy yielding halorespiration (3,4). The occurrence of RDH under methanogenic and sulfate reducing conditions has been observed in a variety of anaerobic mixed cultures (3,4), but only one bacterial strain (*Dehalococcoides* strain CBDB1) capable to couple energy conservation with RDH of CBs (≥ 3 chlorine substituents) has been isolated so far (5,6). RDH generally results in the transformation of higher chlorinated CBs but may lead to an accumulation of lower chlorinated CBs such as monochlorobenzene (MCB) under strongly reducing conditions. Preliminary indications for RDH of MCB have been presented (7). Kaschl et al. suggested that anaerobic MCB degradation may lead to significant stable carbon isotope fractionation and applied compound-specific stable isotope analysis CSIA to further indicate in situ biodegradation of MCB in an anoxic aquifer (8). Recently, complete mineralization of MCB has been proven using stable isotope tracer techniques which enabled very sensitive detection of low rate degradation processes (9).

CSIA has been established for monitoring the biodegradation of pollutants at contaminated field sites (10). This method was successfully applied for BTEX (11-14), fuel oxygenates (15,16) and chlorinated ethenes (17-20) but, to our knowledge, beside the work of Kaschl et al. (8) no further field studies exist for CBs. Moreover, most studies were conducted to characterize degradation of single contaminants instead of looking at complex scenarios involving sequential degradation mechanisms, multiple contaminants and/or various degradation pathways.

Microbial degradation of organic contaminants is associated with isotope fractionation, leading to an enrichment of heavier isotopes in the residual non degraded fraction. The magnitude of isotope fractionation mainly depends on the reaction mechanism. As shown for RDH of chlorinated ethenes (19,21) and trichlorobenzenes (TCB) (22), degradation of CBs should also be associated with significant isotope fractionation, if the reduction of a Cl-C-bond is involved. Similar to anaerobic BTEX degradation, the anaerobic oxidation of the benzene ring for which cleavage of a C-H-bond is expected should lead to isotope fractionation (23,24). However, if the fate of a contaminant is simultaneously controlled by its production (associated with depletion in ^{13}C) and further degradation (leading to enrichment in ^{13}C), as expected from sequential RDH, the isotope enrichment might be masked due to opposite isotope fractionation limiting the use of the Rayleigh approach for contaminants (17,19).

At the study site, release of CBs resulted in a severe contamination of the groundwater. It is hypothesized that RDH and/or anaerobic oxidation govern the removal of CBs under anoxic conditions in the aquifer. Since active remediation technologies were recognized as technically and economically not feasible (25), this study aimed at evaluating the efficacy of natural attenuation (NA) as remedial option.

For this purpose, in accordance with guidelines provided by the US-EPA (26), we developed an integrative approach including (i) assessment and monitoring of CB degradation and plume stability by means of CSIA and tracer studies under field and laboratory conditions as well as (ii) characterization of hydrogeochemical conditions governing in situ biodegradation applying multivariate statistics. Special attention was put in interpreting MCB degradation since its accumulation seems to be the limiting factor to apply NA as remediation strategy. To overcome complications associated with the Rayleigh concept for interpretation of the isotope data, an isotope balance was calculated to assess biodegradation of CBs.

MATERIAL AND METHODS

Study Site and sampling. The field site is located at a former chemical plant where the production of mostly pesticides caused an intensive release of chlorinated organic contaminants to the subsurface. The direct source zone characterized by high loads of contaminants and dense nonaqueous phase liquid (DNAPL) was completely encapsulated and hydraulically disconnected from the surrounding natural groundwater system to prevent further release of contaminants. The initial contaminant pattern dominated by higher chlorinated benzenes is preserved inside the containment whereas a plume mainly consisting of MCB and DCB with total concentrations up 2840 µg L^{-1} has developed. A detailed site description is given in supporting information (SI) (section 1.1).

Groundwater samples for hydrogeochemical and isotope analyses were taken in 2005 and 2006 from 22 monitoring wells (Fig. 1). The sampling, extraction procedure and analytical methods are described in SI. A table summarizing the geochemical data collected during sampling in 2005 is given in SI (Tab. S2).

Chemicals. The [$^{13}C_6$] MCB was purchased from Chemotrade Leipzig (Germany) with chemical and isotopic purity of > 99%. All other chemicals used were obtained in p.a. quality or higher.

Field study with in situ microcosms. In February 2005 a field experiment was performed using an in situ microcosm test system (BACTRAP) as described elsewhere (9,27-30). Briefly, Bio-Sep® beads were loaded with [$^{13}C_6$] MCB to a concentration of about 100 mg g^{-1} beads. The in situ microcosms were deployed in 6 different wells at the low (H, L), medium (D, E) and high contaminated (A, B) area of the plume in the lower strictly anoxic part of the aquifer (Fig. 1 and Fig. S1). After 72 days in situ microcosms were removed from the aquifer and the carbon isotope signature of total lipid fatty acids (TFLA) was analyzed. Detailed information about the extraction procedure and analytical methods can be found in SI (section 1.4).

Laboratory study with enrichment cultures. Material of in situ microcosms amended with natural abundance MCB as described above were used as inoculum for the laboratory enrichment cultures. From each selected well (A, B, D, E, H, L) four enrichment cultures were prepared in 38 ml vials. Each

vial finally contained approx. 27 ml of groundwater and 4-6 Bio-Sep® beads. MCB was added to the vials: always two cultures were amended with 1 µl natural abundant and another two with [$^{13}C_6$] MCB. Cultures were incubated stationary at 20°C in the dark and were sampled at regular time intervals to determine the carbon isotope signature of mineralization products (CO_2, CH_4). A more detailed description of the methods is given in SI (section 1.5).

Determination of $\delta^{13}C$ values for the total chlorinated benzenes. The isotope signatures of individual CBs ($\delta^{13}C_i$) were analyzed by GC-C-IRMS and are given in δ-notation (per mill) (for details see SI, section 1.6).

The isotope signature (per mill) of the total CBs ($\delta^{13}C_{CB}$) was calculated by multiplying the molar concentration of each compound (C_i) with its respective carbon isotope signature ($\delta^{13}C_i$), adding all contributions and dividing by the total molar concentration of all chlorinated benzenes (C_{CB}) (Eq. 1).

Equation (1) $$\delta^{13}C_{CB}[‰] = \frac{\sum (C_i * \delta^{13}C_i)}{C_{CB}}$$

In this paper we refer to the term *isotope balance* to clearly distinguish between the calculated isotope signature of the total CBs ($\delta^{13}C_{CB}$, isotope balance) and the directly measured isotope signatures ($\delta^{13}C_i$) of the single species of CBs, such as MCB and DCB isomers.

The uncertainty associated with the isotope balance was calculated based on the error propagation using the standard deviation of each chlorobenzene species (Eq. 2).

Equation (2) $$\Delta_{tot}\delta_{stdev} = \frac{\sqrt{\sum \left(C_i * \Delta\delta^{13}C_i\right)^2}}{C_{CB}}$$

For all samples the standard deviation for the values of the isotope balance was ≤ 0.4 ‰.

Principal Component Analysis. Principal component analysis (PCA) was used to condense the hydrogeochemical and isotope data into a reduced number or orthogonal linear combinations and to gain insight into the relationship between variables. The numerical data matrices were converted and the correlation analyses carried out using the program R (R: Copyright 2005, The R Foundation for Statistical Computing Version 2.1.1). PCA was performed on the correlation matrix applying the

logarithmic transformation to meet normality requirements. Two data sets were subjected to PCA: (i) the hydrogeochemistry data (Tab. S2), isotope composition of MCB and of total CBs corresponding to the year 2005 (Tab. S4), as well as (ii) concentrations of MCB and DCB isomers, isotope composition of MCB and the total CBs corresponding to years 2005 (Tab. S4) and 2006.

RESULTS AND DISCUSSION

In situ microcosms. The patterns of TFLA extracted from in situ microcosms are provided for one representative well located at the low (H), medium (E) and high contaminated (A) area of the plume (Fig. S1). All samples showed significant amounts of saturated hexadecanoic (C16:0) and octadecanoic (C18:0) acids as well as the unsaturated hexadecenoic (C16:1) and octadecenoic (C18:1) acids. Further, an octadienoic acid (C18:2) was identified in all samples. Comparison of the three samples revealed only minor differences in the fatty acid (FA) compositions. While sample H was dominated by C18:0, the most abundant FA in the other two samples was a C18:1 isomer. In sample A, octadecanoic acid was one order of magnitude less abundant compared to E and H (Fig. S1). TLFA profiles showed low taxonomic value similar to previous findings (27,30). The total concentrations of TLFA varied between 4000-12000 pmol per microcosm (data not shown) suggesting a significant microbial colonization in the bead material similar to previously published results (27).

PLFA may offer a sensitive measure to characterize viable bacterial community structures whereas TLFA fraction can additional comprise lipids of dead biomass (31-33). Basically, the microbial community trapped on in situ microcosms may consist of both viable and non-living organisms as well as degraders and non-degraders. Therefore extraction of TLFA was favored over PLFA to investigate more sensitively the incorporation of ^{13}C into total biomass as an indicator for in situ activity of the bacterial community.

Compared to unlabeled controls ($\delta^{13}C_{FAME}$ = -30 ± 5 ‰, data not shown), the TLFA methyl ester fraction of labeled samples showed a clear enrichment of ^{13}C up to 4500 ‰ in individual fatty acids (Tab. 1). Fatty acids with 16 carbons generally represented the highest enrichment in ^{13}C and

unsaturated FA were higher labeled than the saturated ones as similarly observed by Nijenhuis et al. (9). FA with odd chain length (C17) showed the highest $\delta^{13}C$ value of 4500 ‰ which corresponded to 6 atom % incorporation, but was only found in sample A in very low quantity. For some FA (C18:2, C18:1) no label was found (-31 to -23 ‰) indicating part of the microbial community were not involved in MCB degradation.

However, the transformation of the labeled carbon from the [$^{13}C_6$] MCB into bacterial fatty acids provided evidence of microbial degradation of MCB under ambient aquifer conditions although an enrichment of ≤ 6 atom percent indicated that microorganisms colonizing the in situ microcosms mainly used other carbon sources than the labeled MCB. In general, the labeling was comparable in all three samples but slightly higher at A suggesting that the presence of a more MCB adapted microbial community correlated with high MCB concentrations.

Laboratory enrichment cultures. To prove the potential for mineralization of MCB by the microflora colonizing the in situ microcosms, cultivation-dependent methods were used in the laboratory. Surfaces are very often required for successful cultivation and pre-incubation in the field may improve the later cultivation in the lab (34). Therefore, material of in situ microcosms, which were directly incubated at the field site, served as pre enriched-inoculum. Application of ^{13}C-labeled substrates in enrichment cultures is one of the few approaches suitable to sensitively detect complete mineralization of a single contaminant in a complex mixture determining the evolution of labeled CO_2 (35). Addition of [$^{13}C_6$] MCB allowed exclusive detection of anaerobic MCB degradation, a supposedly slow process (9).

After 197 days highest enrichment of ^{13}C in CO_2 was observed for the samples from well L with values of 629 ± 85 ‰ and lowest for wells H and D with values of 91 ± 59 ‰ and 130 ± 40 respectively. Analyses of enrichment cultures from A, B and E showed comparable results with $\delta^{13}C_{CO2}$ of 250 ± 91 ‰, 222 ± 19 ‰ and 296 ± 122 ‰, respectively (Tab. S3). Labeled methane could not be detected indicating that MCB degradation was not coupled to methanogenesis. All unlabeled controls showed no ^{13}C enrichment in CO_2 during the course of the experiment.

The extent of MCB mineralization was calculated according to the method of Morasch et al. (35) based on the ^{13}C-CO_2 production during the course of the experiment in respect to the total CO_2 initially analyzed from the groundwater of the respective wells (Tab. S3). Mineralization rates varied between 0.1 to 1.1 nmol per day indicating very slow MCB mineralization underlining the difficulties associated with cultivation of such organisms. The results are in good agreement with data obtained from Nijenhuis et al. (9) for a similar experiment at another field site. An electron balance to link MCB degradation to iron or sulfate reduction was not feasible due to too high background concentrations of potential electron donors (Tab. S2). In all samples MCB mineralization was further maintained over time up to 1000 days (data not shown). However, the underlying pathway could not be elucidated.

In summary, both the in situ microcosm and laboratory investigations confirmed anoxic degradation of MCB, providing proof of assimilation of MCB-derived carbon and mineralization of MCB. In situ microcosms provide a very promising tool to directly test in situ biodegradation of recalcitrant contaminants within reasonable time and this technique opens prospects for detailed analysis of the microbial key-players in future.

Isotope balance computation. CSIA of MCB and DCB was performed to gain information on in situ biodegradation within the plume and to investigate the relevance of degradation processes at the field site. Presence of these contaminants in the plume is presumably related to RDH of higher chlorinated benzenes and HCH which were initially spilled and which are still present inside the containment (SI, sections 1.1, 2.4).

The isotope signatures of MCB and DCB dissolved in the groundwater inside the containment were in the range of -27.7 to -29.7 ‰ and -24.0 to -27.5 ‰, respectively. In the plume DCBs showed a significant enrichment of ^{13}C compared to the containment and the isotope signatures were in the range of -25.5 to -15.6 ‰, -28.1 to -22.3 ‰ and -28.1 to -22.0 ‰ for 1,2-; 1,3- and 1,4-DCB, respectively (Tab. S4, Fig. S5). Assuming that due to their relatively low concentrations, RDH of TCB is not a relevant process within the plume and other sources for DCB production are lacking (SI, section 2.4), this high variability is supposedly related to microbial DCB degradation although no systematic

correlation between concentrations and isotope signatures was observed. With increasing distance from the containment an enrichment of ^{13}C in the residual DCB fraction up to 4-5 ‰ compared to the source area was observed. Nevertheless, the highest isotopic shift (10 ‰ for 1,2-DCB and 4 ‰ for 1,3-DCB) was recorded in the area defined as contaminant source of the plume close to wells A, B, D suggesting that this part of the aquifer is probably favorable for RDH of DCB possibly associated with an accumulation of MCB. Further, our data supported the hypothesis of a preferential degradation of 1,2- over 1,3- and 1,4-DCB presumably due to higher yields in Gibbs free energy (36) since lowest concentrations were determined for 1,2-DCB accompanied by the highest enrichment in ^{13}C and accordingly 1,4-DCB showed highest concentration and lowest isotope fractionation.

Under anoxic conditions RDH is the known degradation pathway for DCB leading to the formation of MCB, which consequently should be depleted in ^{13}C. Indeed in most of the wells the isotope signature of MCB was isotopically lighter compared to DCB. Theoretically, also a direct mineralization of DCB to CO_2 is feasible, but so far this has only been described for aerobic biodegradation (3). The high abundance of MCB in the plume further indicated that apparently RDH of DCB and probably TCB led to the formation of MCB. Since no significant amounts of higher chlorinated benzenes were detectable in the plume, we suppose that the isotope signatures of DCB were only affected by its degradation.

The isotope signatures of MCB in the plume ranged between -29.6 ‰ and -25.3 ‰. Highest enrichments were found at the N and SE fringe of the plume (Fig. S5), where MCB concentrations are rather low. The ^{13}C-enrichment in combination with low MCB concentrations indicated MCB biodegradation in this aquifer zone. In the central and western part, presumably representing the former centerline of the plume, $\delta^{13}C$ values of MCB were more depleted indicating microbial production of MCB (Tab. S4, Fig. S5).

As previously suggested, two main mechanisms for MCB degradation should be considered: (i) RDH of MCB to benzene and its subsequent degradation or (ii) anaerobic oxidation of MCB which in this case would serve as electron donor (7,9,37). Similar to RDH of TCB or anaerobic degradation of benzene, it is expected that microbial degradation of MCB under anoxic conditions would also lead to

significant isotope fractionation (22-24). At the anoxic aquifer of the study site the situation is more complex: MCB could be the product of DCB dehalogenation and simultaneously be subjected to microbial degradation. Both processes are associated with an opposite isotope fractionation. The RDH of DCB may result in isotopically lighter MCB and the MCB degradation would lead to an enrichment of ^{13}C in the residual MCB fraction. For this reason the isotope signature of MCB may reflect both processes, production of MCB from DCB as well as MCB biodegradation and the Rayleigh concept is not applicable straight forward to quantify biodegradation of MCB.

In order to investigate whether or not degradation of MCB occurred, an isotope mass balance including all CBs detected was performed based on two main assumptions: transformation of DCB to MCB *via* RDH which is most probably associated with isotope fractionation would not influence the cumulative isotope composition of all CB ($\delta^{13}C_{CB}$) and consequently only further MCB degradation would affect the isotope balance. Therefore significant enrichment of $\delta^{13}C_{CB}$ can be considered as indicator for MCB biodegradation. Regardless of the extent of isotope fractionation of individual contaminants upon formation and degradation, the cumulative isotope composition becomes enriched upon the degradation process. This assumption is valid even if a particular degradation reaction in the reductive sequence is not associated with significant isotope fractionation. However, at least one reaction step of the reduction sequence should be linked to isotope fractionation to document biodegradation. The isotope mass balance approach is corrupted, if alternative degradation pathways, for example mineralization of a DCB isomer without formation of MBC, occur in parallel and affect concentration or isotope signature of one member of the hypothetical sequence. In this case DCBs and MCB will not form a complete reaction sequence and the enrichment of individual species reflects degradation. However, isotope enrichment of DCBs will suggest in situ biodegradation in any case. In addition, the isotope enrichment of MCB at the plume fringes compared to the source areas will clearly indicate in situ degradation. Both, the mass balance approach as well as the individual evaluation of MCB may provide evidence that NA processes governing MCB concentration. In addition, different contaminants may vary significantly concerning their physico-chemical properties which could cause a

preferential transport of contaminant species between source and down gradient the plume. For DCB and MCB no considerable differences concerning their retardation are expected, since adsorption coefficients for both are almost identical (25).

The spatial distribution of concentration and respective carbon isotope signatures for the total CBs ($\delta^{13}C_{CB}$) is presented in Fig. 1. The $\delta^{13}C_{CB}$ values ranged between -27.2 ‰ up to -24.8 ‰. The lowest $\delta^{13}C_{CB}$ value was found for well A, located nearby the containment and defined as the centre of the plume due to highest contaminant concentrations. The decrease in total CB concentration was associated with significant enrichment of ^{13}C by ≥ 1 ‰ (Fig. 2) indicating in situ biodegradation. Highest ^{13}C-enrichment of total CBs was mainly observed in the west (well O and N) and north (well J) of the plume suggesting removal of CBs from the system due to biodegradation (Fig. 1, 2). Overall, with the exception of well B and I, the data obtained from the isotope balance computation indicated a destructive removal of total CBs from the aquifer and demonstrated that chlorobenzene degradation is a relevant process in most parts of the plume. Abiotic processes such as dilution were generally not expected to cause considerable isotope effects, but they can influence the contaminant concentration (38,39) (Fig. 2).

The source zone (A, D, B) and the western fringe of the plume, where DCB is still present in higher amounts, were characterized by more enriched $\delta^{13}C_{DCB}$ and $\delta^{13}C_{CB}$ values suggesting microbial degradation of DCB leading to the formation of MCB more depleted in ^{13}C. MCB isotope signatures did not reflect MCB degradation in the western part of the plume. Nevertheless, the isotope balance of total CBs showed ^{13}C enrichment suggesting that chlorobenzenes are destructively removed from the aquifer. At the SE fringe, higher $\delta^{13}C_{MCB}$ values directly indicated MCB degradation. DCB is not very abundant at this part of the aquifer and consequently significant formation of MCB due to DCB degradation is unlikely.

Statistical analysis of the field data. Principal Component Analysis (PCA) was used to highlight relations between hydrogeochemical patterns of various sampling locations and to support delineation of prevailing biogeochemical processes at the contaminated groundwater system (40,41).

PCA permitted to identify factors driving the separation between clusters and consequently highlighting geochemical variables associated with changes in the isotopic signatures. In the hydrogeochemical analysis of year 2005, the percent of variance within the data accounted for by the first (PC1) and second principal component (PC2) was high (55 %; Fig. 3). Scores of PC1 correlated positively to $\delta^{13}C_{MCB}$, total organic carbon (TOC), electric conductivity, ferrous iron, methane and $\delta^{13}C_{CB}$ (loadings > 0.3) and negatively to total CB and MCB concentrations. This emphasizes the significance of these variables in distinguishing clusters of sampling locations. Moreover, isotopic composition values of both MCB and CBs were negatively correlated with their concentration values (Pearson's product-moment correlation, $P<0.05$) which supported the results of the isotope balance illustrating stable isotope fractionation as a function of microbial degradation. Sampling locations experiencing degradation activity (cluster II and IV) could be distinguished from apparently less active locations (cluster III) or locations where the activity could hardly be detected (cluster I). Overall, higher PC1 and PC2 scores indicated higher biodegradation activity in a well (Fig. 3).

Interestingly, four clusters could be distinguished by PCA corresponding to geographical zonation: (i) heavily contaminated area (wells A, B, C, D), (ii) SW fringe (G, H, N, O), (iii) SE fringe (wells E, F, I, L) and (iv) northern fringe of the plume (wells J, M) (Fig. 3, Fig. 2 for location of the wells). Cluster I representing the high contaminated region of the plume is consequently correlated with high contaminant concentrations. Cluster II grouped positively along PC2, which is associated with manganese, sulfate and $\delta^{13}C_{CB}$ emphasizing relatively higher values for these variables at the SW fringe. Although enrichment in ^{13}C indicating biodegradation has been observed in this region, a linkage between anaerobic oxidation and presence of sulfate or manganese could not be ruled out. Samples from the SE fringe of the plume (cluster III) grouped close to the origin and thus show intermediate hydrogeochemical patterns with no extreme values in the variables taken into account for PCA.

Alternatively, sampling locations of the northern fringe (cluster IV) clustered positively along PC1 and displayed relatively higher values of ferrous iron, methane, TOC and electric conductivity. At this area, MCB is present > 60% of total CB and isotope enrichment of CB and MCB is likely to mirror MCB degradation. Because $\delta^{13}C_{MCB}$ values were positively correlated with ferrous iron, TOC and electric conductivity (P<0.05), a linkage between these geochemical indicators and degradation activity may exist. Indeed, TOC may serve as a source of electron donors to support RDH of CBs. Moreover, positive correlation of ^{13}C enriched MCB with increased ferrous iron concentrations may suggest anoxic oxidation of MCB during ferric iron reduction.

Caution should be taken classifying sampling locations, as PCA directly reflects the limits of the methodology on which each variable integrated in the analysis has been derived. For instance, samples belonging to cluster I were collected in a highly contaminated part of the plume and showed no shift in $\delta^{13}C$ values of MCB or of total CBs. This could be used as indicator for recalcitrance to biodegradation. However, CSIA suggested DCB degradation due to relatively high isotopic enrichment accompanied by MCB formation (Tab. 3, Fig. S5). The significant DCB enrichment was masked in the isotope balance possibly due to the accumulation of relatively ^{13}C depleted MCB which may point to RDH of DCB. Moreover, the results of the in situ and laboratory tracer studies showed that in situ biodegradation of MCB is feasible under the ambient aquifer conditions, but could not be determined by means of CSIA. This emphasizes the interest of combining several tools of various sensitivity levels to gain evidence of in situ degradation.

Overall, the PCA results underlined the hydrogeochemical heterogeneity of the investigated field site. Delineated clusters of sampling locations showed various hydrogeochemical trends and corresponded to defined zones of the contamination plume, which putatively differ with respects to their potential and development of CB degradation activity. However, causality or mechanistic interpretation could often hardly be proved based on statistical analysis of the field data alone. Together with other lines of evidence, this delineation may be relevant in terms of risk assessment when coupling information about

dominant contaminant flow patterns with zonation of potential biogeochemical characteristics at the plume scale.

To further confirm that monitored natural attenuation might be suitable as remediation method at the study site stability of the plume as well as of NA processes needs to be demonstrated (26). The comparative analysis of contaminant and isotope signature patterns of sampling locations in year 2005 and 2006 by PCA did not reveal substantial changes (Fig. S3). Additionally, the temporal evolution of contaminant concentration (2000-2007) and corresponding isotope signature (2005-2007) were analyzed in more detail for wells H, F, L at the southern fringe of the plume (Fig S4-6). In all three wells, concentrations varied significantly while the isotope signatures remained relatively stable over time. Although minor fluctuations within the data occurred, the constant enrichment of heavy isotopes compared to central parts of the plume indicated that the biodegradation potential was maintained over time demonstrating plume stability presumably controlled by microbial activity (see also SI, section 2.5).

Implications for environmental studies and site management in terms of natural attenuation

This study aimed at evaluating the efficacy of NA applying an integrated approach. Multivariate statistics permitted to delineate various geochemical zones within the plume associated with microbial degradation suggesting variability of processes involved in contaminant removal underlining the complexity and heterogeneity of the field site. PCA supported the results obtained by the isotope balance and enabled a combined analysis of geochemical and isotope data to evaluate the in situ biodegradation potential. To explore significant spatial and temporal changes of the hydrogeochemical patterns statistical methods are helpful and CSIA can reveal if biodegradation controls plume stability. Such methods can be easily implemented into a monitoring campaign. At the investigate area plume stability was demonstrated over time. Major changes in environmental conditions that may reduce the efficacy of the NA processes especially that of in situ biodegradation, are not likely to occur at the field site.

As basically required by authorities and scientists we presented an integrative concept encompassing multiple lines of evidence for NA of recalcitrant contaminants such as CBs at contaminated complex subsurface environments (26,42). At many field sites numerous contaminants are present and different

mechanisms may govern the fate of pollutants in situ. Especially at such complex systems characterized by multiple contaminants, sequential degradation and/or different potential pathways one single method can not elucidate all relevant biogeochemical processes. Therefore, coupling of several techniques may lead to a more robust assessment and allows verifying plausibility of lines of evidence for in situ biodegradation processes. This further results in complex sets of data which can be treated more easily by statistical methods to interpret the relationship between certain parameters and to analyze the influence as well as temporal changes of hydrogeochemical factors on in situ biodegradation without substantial additional monitoring costs (40,41).

Even for recalcitrant contaminants (e.g. MCB) with unknown degradation pathways, an appropriate methodology may provide evidence of in situ biodegradation. An integrative approach consisting of hydrogeochemical analysis combined with novel stable isotope tools and multivariate statistics may substantially support identification of mechanisms and main biogeochemical factors associated with contaminant removal. At the complex chlorobenzene contaminated field site evaluation of NA potential showed that CBs are subjected to microbial degradation leading to sustainable removal of pollutants from the groundwater.

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Supporting Information Available

Detailed description of the field site, sampling, experimental set up, analytical methods, hydrogeochemical data, chromatograms of in situ microcosm analysis, $\delta^{13}C_{CO2}$ of laboratory enrichment cultures, isotope analysis of chlorinated benzenes, plume stability is available free of charge via the Internet at http://pubs.acs.org.

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FIGURES

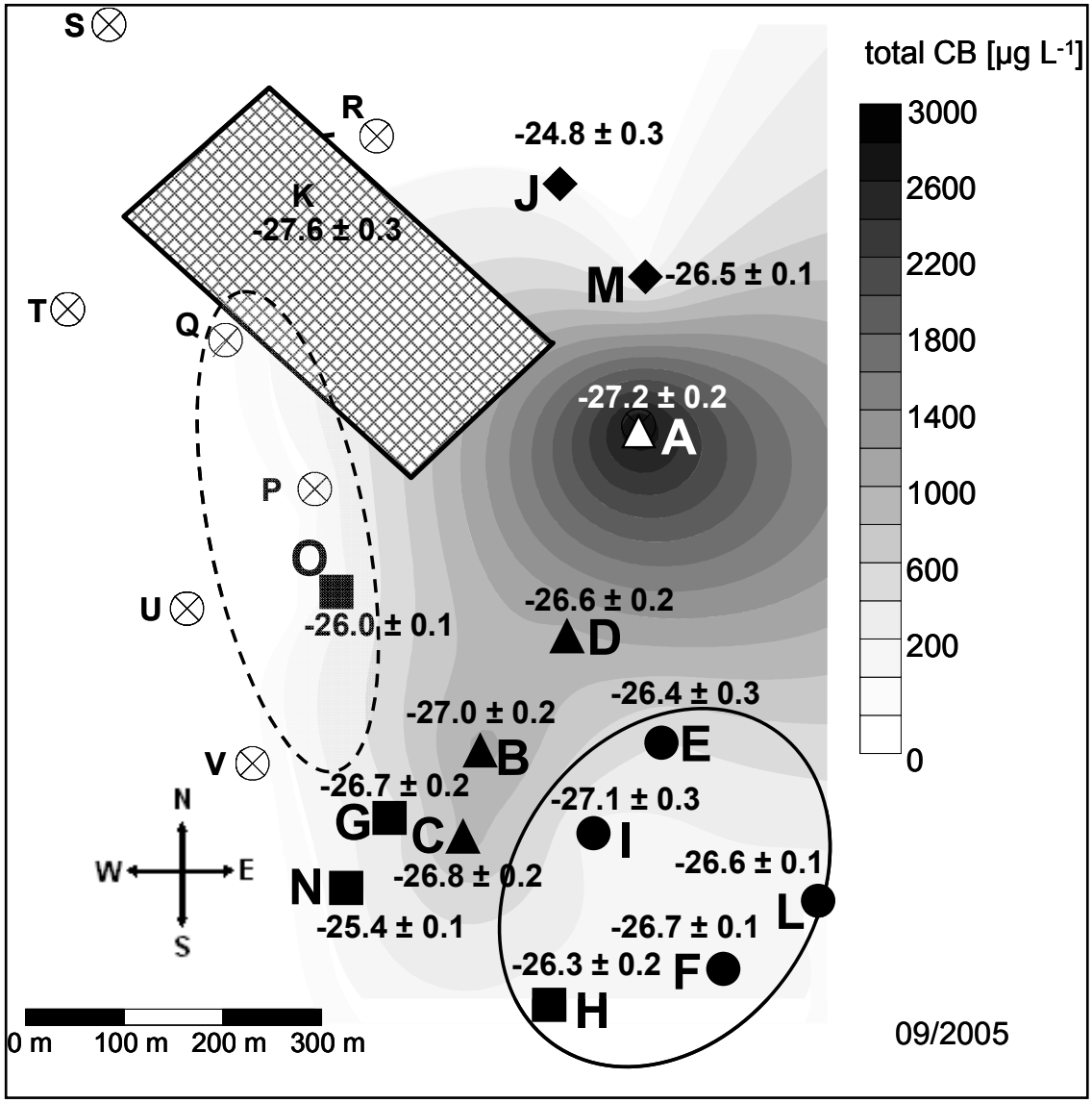

Fig. 1: Concentrations [µg L^{-1}] and carbon isotope signatures [‰] of total chlorinated benzenes (numbers) at the study site for sampling in 2005. The letters indicate the name of the wells and the rectangle in diagonal crosses surrounds the area of the containment, which is hydraulically disconnected from the plume. Additionally the dashed (- - -) black line shows the area were the ratio between MCB and DCB is < 0.3 and the solid (——) line indicates where the ratio between MCB and DCB is > 0.9. The symbols indicate the samples which belong to a common cluster as obtained by PCA: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M) (see fig. 3). Wells which were not considered for PCA are indicated by ⊗.

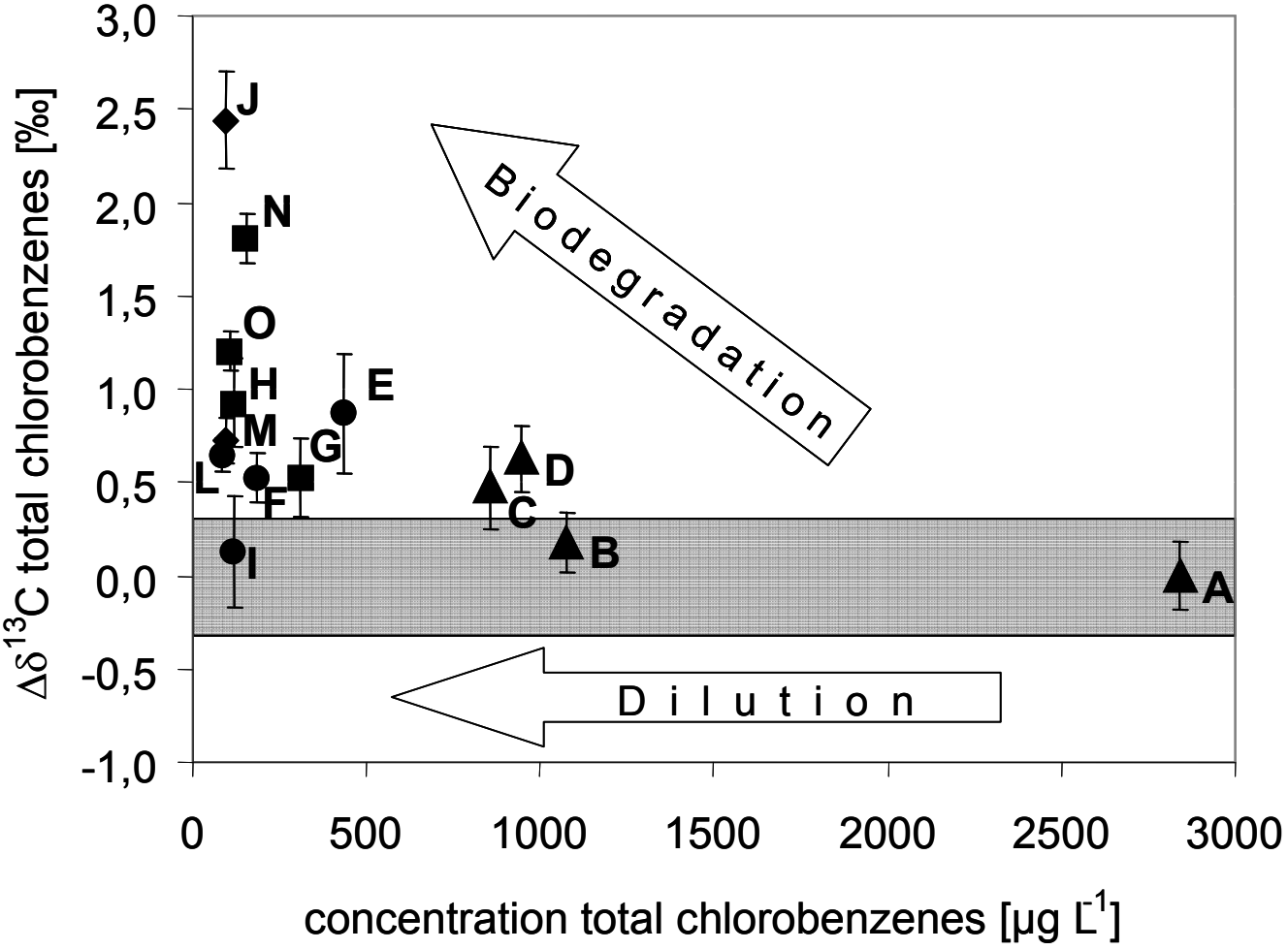

Fig. 2: Correlation between concentration [µg L^{-1}] and isotopic enrichment [‰] of total chlorinated benzenes. The symbols indicate the sampling locations which belong to a common cluster as obtained by PCA: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M) (see fig. 3).

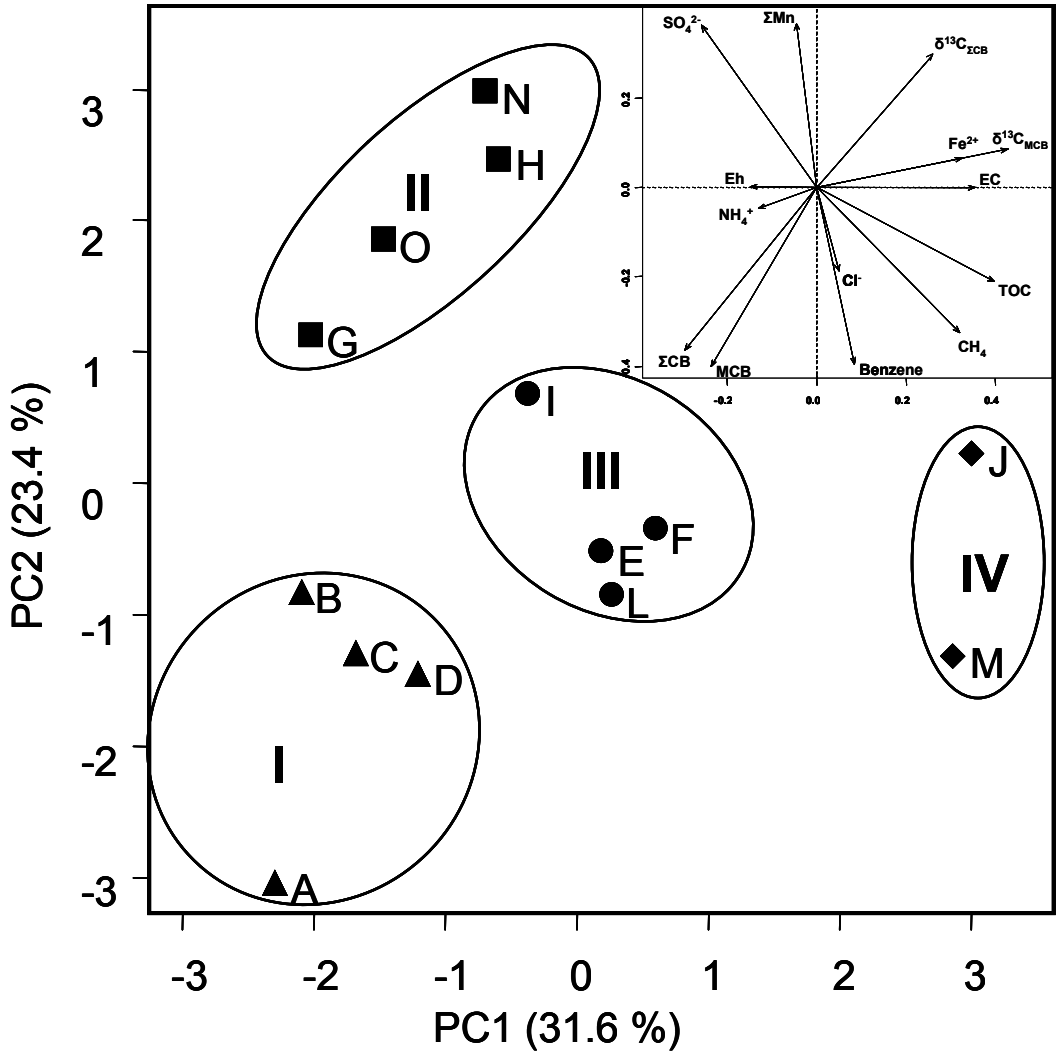

Fig.3: PCA ordination plot of geochemistry, contaminant concentrations, carbon isotope composition of MCB and total CBs in groundwater samples obtained during sampling in 2005. Values on the axes indicate % of total variation explanation by the corresponding axis (PC1, principal component axis 1; PC 2, principal component axis 2). The symbols indicate the samples which belong to a common cluster: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M). The figure in the upper right corner of the plot depicts the relative contribution of the descriptors in the reduced space. Description vectors correspond to: $\delta^{13}C_{\Sigma CB}$, isotope balance of total CBs; $\delta^{13}C_{MCB}$, carbon isotope signature of MCB; Fe^{2+}, ferrous iron; EC, electric conductivity; TOC, total organic carbon; CH_4, methane; Cl^-, chloride; MCB, monochlorobenzene; ΣCB, total chlorobenzenes; NH_4^+, ammonium; Eh, redox potential; SO_4^{2-}, sulphate; ΣMn, total manganese.

TABLES

Tab. 1: Carbon isotope signature of TLFA methyl esters $\delta^{13}C_{FAME}$ [‰] extracted from in situ microcosms amended with [$^{13}C_6$] MCB and incubated in the contaminated aquifer at the study site.

Well [C_{MCB} **in µg** L^{-1}]	**A** [1400]	**E** [430]	**H** [120]
C16:1	2600	2000	2000
C16:1	nd	nd	2900
C16:0	1600	70	10
iC17:0	4500	nd	nd
C18:2	-28	-31	-31
C18:1	-28	-31	-31
C18:1	360	43	1
C18:0	50	-25	-31
C21:0	-23	-26	-30

nd - not detectable

Supporting Information for publication in ES&T

Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers

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Content

1 Materials und Methods

1.1 Field site

History. The field site is located at a former chemical plant in Germany where the production of mostly pesticides caused an intensive release of chlorinated organic contaminants to the subsurface. After closing down of the factory all facilities were demolished and 1998 the direct source zone with high loads of contaminants and dense nonaqueous phase liquid (DNAPL) was completely encapsulated and hydraulically disconnected from the surrounding natural groundwater system to prevent further release of contaminants. During the past, active water supply wells located at about 1.5 km in the south of the investigated field site caused a hydraulic gradient, which controlled the distribution of the contaminants. After shutting down the supply wells in 1990, the groundwater flow changed into SW to NE direction creating an overall movement of the contamination plume towards the east. In addition, in central parts of the plume the groundwater flow is hydraulically controlled by groundwater subtraction wells, whereas the natural groundwater flow at wells F, G, L at the southern fringe of the plume is not affected by hydraulic control wells. The initial contaminant pattern dominated by higher chlorinated benzenes is preserved inside the containment whereas the plume mainly consists of MCB and DCB (see also 2.4).

Geology. The aquifer consists of layered Quaternary fine to coarse sandy materials of about 20 m thickness which are overlain by up to 2 m clay deposits. In the area of the former production site additional 6 to 8 m of refilled material are on top of the soil profile. The basis of the aquifer is characterized by impermeable Tertiary clays (1).

Geochemistry. In all zones of the aquifer the pH was rather neutral and constant ranging from 6.7 to 7.4. Oxygen concentrations were always below detection limit and the redox potential was generally between -110 and -150 mV (except of well G with -94 mV) indicating anoxic conditions in all parts of the plume. The conductivity was relatively high ranging between 1117 and 1468 µS cm^{-1}, probably due to the entry of brackish water from the canal north of the plume which is influenced by the tide.

Today, the main contaminant of the plume is chlorobenzene with concentrations up to 1400 µg L^{-1} in well A which is defined as the source zone of the plume. The MCB plume is still approximately 1 km long (N-S) and several hundreds of meters wide (W-E). The exact boundaries of the plume in the East are not known, because monitoring wells in this region are missing. The MCB concentration in wells T, U, V is below detection limit (0.1 µg L^{-1}) indicating the western boundary of the MCB plume. The wells A, B, C, D are located in the former centerline of the plume and therefore this area is still characterized by high MCB concentrations of 1400, 630, 590 and 560 µg L^{-1} respectively (Fig. S5).

Dichlorobenzenes (DCBs) are also present with maximum amounts of 1442 µg L^{-1} for total DCB in well A. The most abundant isomer is 1,4-DCB which in general accounts for more than 50% of total DCB, followed by 1,3-DCB (15-40% of total DCB) and 1,2 DCB (< 10% of total DCB). This can be explained by the fact that DCB isomers with the most adjacent chlorine substituents will be preferably used during reductive dehalogenation (RDH) (2). In anoxic environments higher chlorinated benzenes are preferably degraded via RDH which often results in an accumulation of lower chlorinated chlorobenzenes. During last 10 years, trichlorobenzene (TCB) only occurred close to the containment in concentration up to 190 µg L^{-1} (well Q), whereas TCB concentration in all other wells was always below 5 µg L^{-1} suggesting that higher CBs have probably become degraded since the isolation of the source area about 10 years ago. Only lower chlorinated benzenes remained in the aquifer. In addition to chlorinated aromatic contaminants benzene was found at the field site with maximum concentrations of 29 µg L^{-1} at the observation well C. In the western part of the plume the benzene concentrations were always below detection limit. Benzene could be originally introduced to the groundwater as contaminant, but could also stem from RDH of MCB (3). However benzene did not accumulate at the field site suggesting further degradation if formed upon RDH of MCB.

Nitrate concentrations were always below detection limit (0.5 mg L^{-1}) but ammonium could be determined in the range of 2 to 21 mg L^{-1} indicating that nitrate is already reduced during microbial degradation and is not a relevant electron acceptor at the site. Ferrous iron was present in all wells (4 to 32 mg L^{-1}) with exception of well K. Highest amounts of ferrous iron were found in the north part (J, M) as well as in the southeast (H, N) of the plume. Lowest iron concentrations were found upstream at the western fringe of the plume representing a low natural background concentration of Fe(II). Increasing ferrous iron concentrations in the plume pointed to iron mobilization possibly due to microbial degradation processes. Sulfate was present in concentrations of 43 to 190 mg L^{-1} whereas sulfide was always below detection limit (0.1 mg L^{-1}). The maximum sulfate concentrations were detected upstream outside the plume and a decrease in concentration at the contaminated area of the aquifer implies sulfate reduction. Iron and sulfate reduction could be feasible at the field site as also suggested by a redox potential of less than -100 mV. Both processes, iron- and sulfate reduction may occur simultaneously leading to precipitation of iron sulfides which hinders the accumulation of both, dissolved iron and sulfide. Thus the concentration of ferrous iron may not represent the true extent of iron reduction. Therefore the iron reduction would be underestimated. Methane was also detected in elevated concentrations of 40-3800 µg L^{-1} with maximum values in the North (J, M) and generally higher values in the eastern part of the plume compared to the background concentrations upstream. This shows that methanogenesis may play a role during microbial degradation in parts of the plume. The total organic carbon

content (TOC) varied between 3.8 to 12 mg L^{-1}. The wells upstream at the western area of the plume showed the lowest TOC content. Maximum TOC concentrations were observed at the northern fringe (J, M) of the plume and towards the east. At the field site chloride was determined in concentrations ranging from 145 to 200 mg L^{-1}. Generally increasing chloride concentrations could be related to reductive dehalogenation, but at the field site background concentrations of chloride were too high for a mass balance (Tab. S2).

1.2 Groundwater sampling

The main samplings took place in September 2005 and 2006. In total 22 wells were selected and groundwater samples were taken using a submergible pump (MP1, Grundfos, Bjerringbro, Denmark) at 15 m below the ground surface. Sampling was performed after purging the wells by removing three volumes of water and parameters like dissolved oxygen, temperature, pH, redox potential and conductivity had stabilized. These parameters were measured using electrodes (OXI 325-A, LF 320, pH 330; Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). For sampling at each well all tubes were exchanged to avoid cross contamination between the wells. The sampling was accomplished by the Technologiezentrum Wasser (TZW Karlsruhe, Germany).

1.3 Geochemical Analysis

All samples obtained during sampling in 2005 were analyzed for concentrations of chlorinated benzenes, benzene and toluene. Additionally ammonium, ferrous iron, manganese, chloride, nitrate, sulfate, sulfide; methane and the sum parameter alkalinity, total organic carbon (TOC) and absorbable organic halogens (AOX) were determined. The chemical analyses were carried out according German standard analytical procedures (Deutsche Industrienorm, DIN) in the laboratories of the TZW Karlsruhe (Germany). All analyzed parameters and the applied methods are listed in Tab. S1.

Tab. S1: Procedures for chemical analysis

Parameter	Abbreviation	unit	procedure
temperature	T	°C	DIN 38404-4-C4
electric conductivity	EC	µS cm^{-1}	DIN EN 27888-C8
pH	pH	-	DIN 38404-5-C5
redox potential	Redox	mV	DIN 38404-6-C6
ferrous iron	Fe(II)	mg L^{-1}	DIN 38406-1-E1
manganese	Mn	mg L^{-1}	DIN EN ISO 11885-E22
ammonium	NH_4^+	mg L^{-1}	DIN EN ISO 11732-E23
chloride	Cl^-	mg L^{-1}	DIN EN ISO 10304-1-D19
nitrate	NO_3^-	mg L^{-1}	DIN EN ISO 10304-1-D19
sulfate	SO_4^{2-}	mg L^{-1}	DIN EN ISO 10304-1-D19
sulfide	S^{2-}	mg L^{-1}	Dr. Lange Test LCW 053
total organic carbon	TOC	mg L^{-1}	DIN EN 1484 H3
absorbable organic halogens	AOX	µg L^{-1}	DIN EN 1485-H14
benzene	Benz	µg L^{-1}	DIN 38409-9-1-F9
toluene	Tol	µg L^{-1}	DIN 38409-9-1-F9
methane	CH_4	µg L^{-1}	GC-FID (headspace)

chlorobenzene	MCB	µg L^{-1}	GC-MS (liquid-liquid extraction, n-hexane)
chlorobenzenes	CBs	µg L^{-1}	GC-MS (solid phase extraction)

1.4 In situ microcosm experiment (BACTRAP)

In February 2005 a field study was performed using an in situ microcosm test system as described in Stelzer et al. 2006 (4). Briefly, for each sampler 0.4 g Bio-Sep beads (University of Tulsa, Tulsa, USA) were loaded with $^{13}C_6$ MCB to a concentration of about 100 mg g^{-1} beads. The in situ microcosms were deployed in 6 different wells at the low (H, L), medium (D, E) and high contaminated (A, B) area of the MCB plume in the lower strictly anoxic part of the aquifer at > 15 m below the ground surface (Fig. 1 of the manuscript, Fig. S1). After 72 days in situ microcosms were removed from the aquifer and extraction of total lipid fatty acids was performed using a single-phase dichloromethane-methanol-water mixture as solvent modified from Bligh and Dyer (5). To obtain fatty acid methyl esters (FAME) the samples were derivatized applying a trimethylchlorosilane (TMCS) methanol mixture (1:8, v:v) for 2h at 70°C (6). After evaporation to complete dryness the FAME fraction was dissolved in *n*-hexane for subsequent analysis. Concentrations of fatty acids were quantified relative to an internal standard (henicosanoic acid C21:0).

GC-MS. For identification and structural characterization of the FAME a Hewlett–Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent, Palo Alto, USA) was used. The carboxylic acid fraction was separated on a BPX-5 column (30 m x 0.32 mm x 0.25 µm) (SGE, Darmstadt, Germany) with a temperature program of 40°C initial temperature [0 min], 20°C min^{-1} to 150°C [0 min], 2°C min^{-1} to 240°C [0 min] and 20°C min^{-1} to a final temperature of 300°C [12 min]. The temperature of the injector was 280°C and samples were injected with a volume of 1 µl and a split ratio of 1:10. Helium was used as carrier gas with constant flow of 2.0 ml min^{-1}. The mass spectra were determined in full scan mode (m/z 50-600). FAMEs were identified by comparison of the retention times with those of an authentic standard mix (bacterial acid methyl ester mix, Sigma–Aldrich, Germany; Fig. S1) and by analyzing their mass spectra. The nomenclature for fatty acids is of A:BωC where A is the number of carbon atoms, B is the number of double bonds and C is the distance of the closest double bond from the aliphatic end of the molecule (unsaturation, ω-nomenclature). The prefix *i (iso)* and *a (anteiso)* refer to terminal methyl branching at ω -1 and ω -2 position respectively.

GC-C-IRMS. A Restek RTX-5 column (60m x 0.32 mm x 0,1 µm) (Restek GmbH, Bad Homburg, Germany) was used for chromatographic separation of FAMEs with helium as carrier gas at a constant flow of 2.0 ml min^{-1}. The following temperature program was applied: 70°C [1 min], 20°C min^{-1} to 130°C [0 min], 2°C min^{-1} to 150°C [5 min], 2°C min^{-1} to 165°C [5 min], 2°C min^{-1} to 230°C [0 min], 20°C min^{-1} to 300°C [5 min]. The temperature of the injector was 280°C and 1 to 5 µl of sample was injected using a split ratio of 1:1.

The determination of isotope signatures up to more than +1000 δ is outside the calibration range of the instrument. Isotope signatures of highly ^{13}C-enriched samples were thus outside the calibration curve for precise δ ^{13}C measurements. The standard deviation increases with higher enrichments and a positive isotope signature therefore is rounded to the second decimal of the value.

1.5 Laboratory study with enrichment cultures

In situ microcosms amended with natural abundance MCB as described before were used as inoculum for the enrichment cultures. After removal of in situ microcosms from the groundwater 0.2 g of Bio-Sep beads were added to 120 ml vials completely filled with groundwater from the corresponding wells. The vials were immediately closed with grey Teflon-coated butyl rubber stoppers and aluminum crimps. After arrival in the laboratory samples were transferred into an anaerobic Coy glove box (96-98 % N_2, 2-4% H_2) and evenly split into 4 sub samples containing groundwater and Bio-Sep beads. From each well (A, B, D, E, H, L) 4 enrichment cultures were prepared in 38 ml vials. Each vial finally contained approx. 27 ml groundwater and 4-6 Bio-Sep beads. After removal from the anaerobic chamber, MCB was added to the vials: always 2 cultures were amended with 1 µl natural abundant and another 2 with [$^{13}C_6$] labeled MCB. Cultures were incubated stationary at 20°C in the dark and were sampled at regular intervals for appearance of the ^{13}C label in products using GC-C-IRMS.

GC-C-IRMS. The carbon isotope ratio analysis of CO_2 from the headspace of the microcosms was carried out isotherm at 40°C using the GC-C-IRMS with a Varian CP-PoraBond Q column (50 m x 0.32 mm x 5 µm) (Varian, Middelburg, The Netherlands). Aliquots of 50 µl of head space samples were injected into a gas chromatograph in split mode, with split ratio of 1:5.

1.6 Stable isotope fractionation analysis

Groundwater samples for isotope analysis of chlorinated benzenes were adjusted to a pH of 10-12 adding sodium hydroxide pellets to inhibit further microbial activity. The samples of about 1 l groundwater were extracted with 2 ml of *n*-pentane and isotope analyses were performed using a GC-C-IRMS system.

GC-C-IRMS. A gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via a Conflow III interface (Thermo Finnigan, Bremen, Germany) to a MAT 252 mass spectrometer (Thermo Finnigan, Germany) was used for all stable isotope analyses. For chromatographic separation a Zebron ZB1 column (60 m x 0.32 mm x 1µm) (Phenomenex, Inc Torrance, USA) was used applying the following temperature program: 40°C [5 min], 4°C min^{-1} to 150°C [0 min], 20°C min^{-1} to 250°C [5 min] with helium as carrier gas at a constant flow of 2.0 ml min^{-1}. The temperature of the injector was 250°C and 1 to 5 µl of sample were injected using split ratios of 1:1 to 1:10.

1.7 Equations

Carbon isotope signatures of chlorinated benzenes, fatty acids and CO_2 were reported in δ-notation (per mill) relative to the Vienna Pee Dee Belemnite (V-PDB) standard (equation 1) (7).

(1) $$\delta^{13}C\,[‰] = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample} - \left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}}{\left(\frac{^{13}C}{^{12}C}\right)_{VPDB-Standard}} \times 1000$$

The Rayleigh equation mathematical describes the relationship between the changes in contaminant concentration (C_t/C_0) and the changes in the isotope ratio (R_t/R_0) during biodegradation through the fractionation factor α.

(2) $$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\left(\frac{1}{\alpha}-1\right)}$$

2 Results

2.1 Geochemistry

Tab. S2: Results from the geochemical and concentration analysis of samples obtained 2005

well	T	EC	pH	Redox	Fe(II)	Mn	NH_4^+	Cl^-	NO_3^-	SO_4^{2-}	S^{2-}	CH_4	TOC	AOX	Benz	Tol	MCB	1,2-DCB	1,3-DCB	1,4-DCB
	°C	µS cm^{-1}		mV	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	µg L^{-1}	mg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}
A*	13.8	1300	6.8	-120	13.3	2.4	12.0	198	< 0.5	111.0	< 0.1	650	9.1	1200	13.0	< 0.5	1400	22	450	970
B*	10.8	1268	6.8	-113	17.4	3.0	10.0	179	< 0.5	102.0	< 0.1	210	7.6	420	4.6	< 0.5	630	18	150	280
C*	15.9	1166	6.8	-120	18.2	3.6	11.0	145	< 0.5	98.2	< 0.1	620	7.9	310	29.0	< 0.5	590	29	95	140
D*	11.2	1245	6.8	-110	16.7	2.7	9.5	171	< 0.5	96.6	< 0.1	850	8.9	410	12.0	< 0.5	560	13	120	250
E*	11.4	1329	6.8	-141	16.7	2.9	10.0	169	< 0.5	120.0	< 0.1	600	11.0	130	8.5	< 0.5	430	< 0.1	0	3
F*	11.1	1349	6.9	-161	16.4	2.8	11.0	171	< 0.5	89.7	< 0.1	820	9.9	80	6.6	< 0.5	170	0	2	10
G*	12.5	1235	6.8	-94	15.3	3.5	11.0	169	< 0.5	123.0	< 0.1	79	7.5	160	0.7	< 0.5	120	17	82	90
H*	10.6	1270	6.7	-130	22.0	4.0	9.0	149	< 0.5	190.0	< 0.1	110	8.3	51	2.6	< 0.5	120	< 0.1	< 0.1	< 0.1
I*	11.0	1314	6.9	-141	16.4	2.4	6.4	177	< 0.5	134.0	< 0.1	160	8.5	55	1.0	< 0.5	120	< 0.1	< 0.1	< 0.1
J*	11.1	1338	6.7	-130	32.3	3.3	9.8	164	< 0.5	65.9	< 0.1	3800	12.0	44	9.5	< 0.5	86	1	2	7
K	13.6	1264	7.0	-118	n.a.	n.a.	n.a	n.a	n.a	n.a	n.a	n.a	6.8	220	16.0	< 0.5	85	8	48	270
L*	13.9	1336	7.1	-117	8.1	1.6	7.5	179	< 0.5	45.2	< 0.1	320	8.7	42	12.0	< 0.5	72	1	2	8
M*	11.3	1468	6.8	-134	30.2	2.9	9.4	202	< 0.5	42.8	< 0.1	1400	12.0	56	18.0	< 0.5	56	1	5	36
N*	10.8	1360	6.8	-121	21.8	4.5	12.0	169	< 0.5	181.0	< 0.1	52	8.0	77	< 0.5	< 0.5	42	7	44	60
O*	10.4	1269	7.0	-136	10.5	3.2	9.5	192	< 0.5	130.0	< 0.1	60	6.1	60	< 0.5	< 0.5	8	1	36	61
P	10.5	1283	7.0	-140	13.1	5.5	21.0	175	< 0.5	111.0	< 0.1	71	7.7	59	< 0.5	< 0.5	7	2	18	48
Q	10.6	1337	7.0	-130	12.1	7.0	17.0	157	< 0.5	120.0	< 0.1	48	7.0	47	< 0.5	< 0.5	2	1	7	29
R	12.3	1323	7.1	-138	9.7	5.3	5.7	189	< 0.5	82.0	< 0.1	150	7.3	21	< 0.5	< 0.5	2	< 0.1	< 0.1	0
S	11.9	1117	7.1	-135	5.8	3.4	2.8	171	< 0.5	166.0	< 0.1	40	5.1	23	< 0.5	< 0.5	1	< 0.1	< 0.1	< 0.1
T	10.8	1287	7.0	-142	13.9	4.4	14.0	175	< 0.5	81.5	< 0.1	68	7.4	17	< 0.5	< 0.5	< 0.1	< 0.1	< 0.1	< 0.1
U	10.5	1312	7.0	-145	13.0	4.4	12.0	201	< 0.5	124.0	< 0.1	54	6.3	14	< 0.5	< 0.5	< 0.1	< 0.1	< 0.1	< 0.1
V	13.7	1180	7.4	-150	4.0	0.3	1.8	180	< 0.5	93.7	< 0.1	120	3.8	6	< 0.5	< 0.5	< 0.1	< 0.1	< 0.1	< 0.1

n.a. not analysed
* data used for PCA

2.2 In situ microcosms

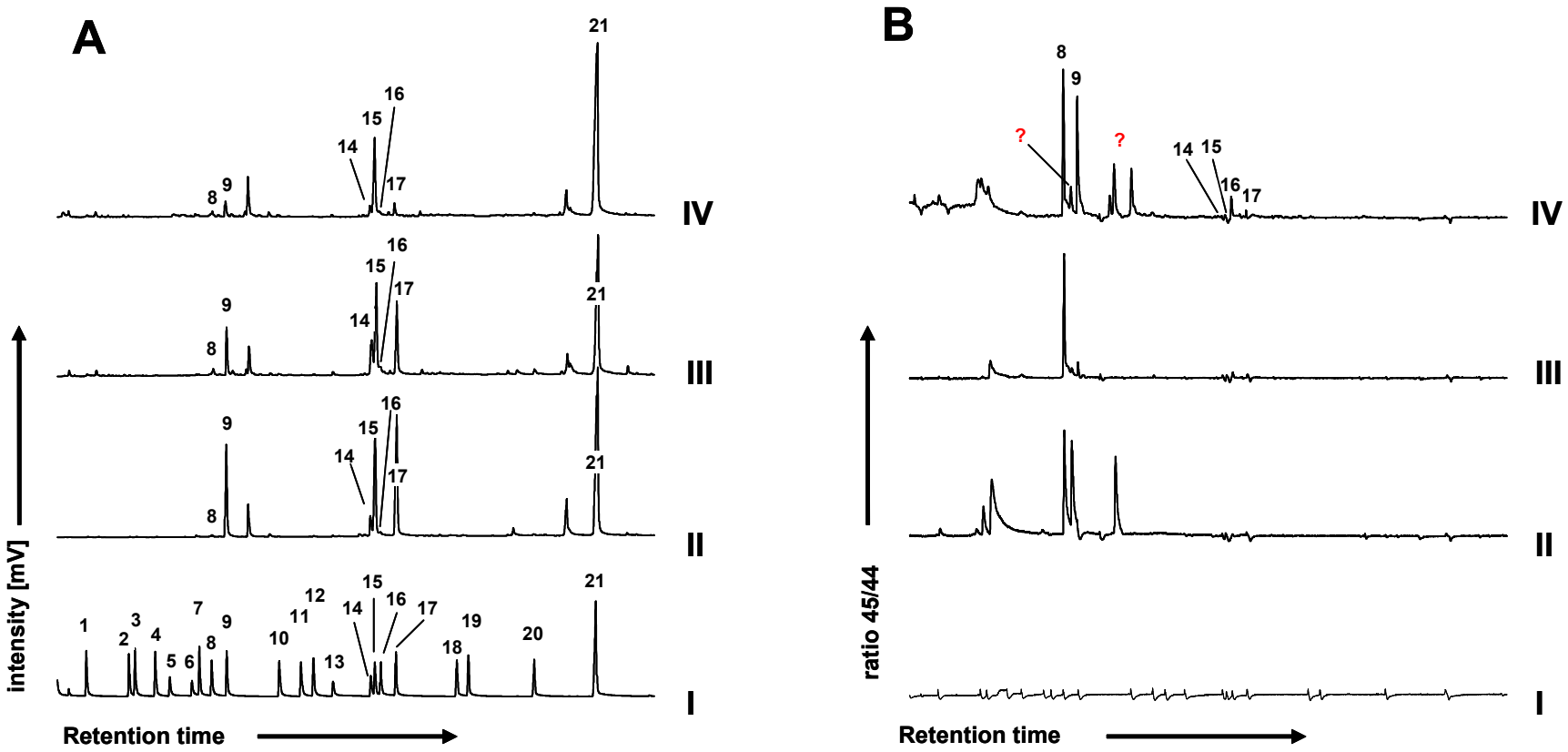


Fig. S1: (A) GC-C-IRMS-chromatograms of the carboxylic acid fraction showing the distribution of fatty acid composition extracted from [$^{13}C_6$]-MCB amended in situ microcosms incubated at the low (II), medium (III) and high (IV) contaminated area of the field site and the bacterial acid methyl ester standard (I). (A) The concentration of the fatty acid methyl esters are displayed by the amount of CO_2 (m/z 44). (B) 45/44 ratio plots indicating ^{13}C enrichment in all the samples (II-IV). In general low abundant fatty acids are highly labeled. For identification of fatty acids see numbers: 1=(C14:0); 2-(iC15:0); 3=(aC15:0); 4=(C15:0); 5=(2-OH C14:0); 6=(3-OH 14:0); 7=(iC16:0); 8=(C16:1); 9=(C16:0); 10=(iC17:0); 11=(C17:0D); 12=(C17:0); 13=(2-OH C16:0); 14=(C18:2); 15=(C18:1c); 16=(C18:1t); 17=(C18:0); 18=(C19:0D); 19=(C19:0); 20=(C20:0); 21=(C21:0). Peaks indicated by question mark (?) could not been identified.

2.3 Laboratory enrichment cultures

Tab. S3: Evolution of $\delta^{13}C_{CO2}$ [‰] in enrichment cultures. The standard deviation displays the isotope composition in parallel microcosms.

time [d]	A	B	D	E	H	L
0*	-19.7 ± 0.9	-20.5 ± 0.9	-21.8 ± 1.1	-11.4 ± 0.2	-22.4 ± 0.6	-22.3 ± 0.6
68	51**	71**	30**	66**	30**	233**
197	250 ± 91	222 ± 19	130 ± 40	296 ± 122	91 ± 59	629 ± 85
C_{CO2}***	4.6	5.7	5.6	6.4	5.2	7.9

* background average of the complete time span (197 days)

** no uncertainty reported because only one analysis (n=1)

*** CO_2 concentrations [mmol mL^{-1}] obtained from carbonate buffer of the groundwater

2.4 Isotope analysis of chlorinated benzenes

Today, the initial contaminant pattern is preserved in the dense non aqueous phase liquid (DNAPL) which still can be found in the direct source zone. As determined by GC-MS analysis the DNAPL is composed of mainly higher chlorinated benzenes (TCB, TeCB, PeCB) and only low amounts of MCB and DCB (Fig. S2, S3). All three DCB isomers are abundant in the same order of magnitude (Fig. S3). The chemical composition of the DNAPL represents the pollutants originally contaminating the aquifer. The lower water solubility of higher chlorinated compounds (e.g. TCB, TeCB) compared to DCB and MCB probably led to a reduced mobility of these compounds in the by-passing water. Consequently, MCB, DCB and small amounts of TCB dissolved in the groundwater and were transported down gradient forming the long CB plume. The contaminants dissolved into the groundwater resulting in a plume of about 1000 m length and 400 m width. Due to low water solubility and stronger retardation of higher chlorinated benzenes such as TeCB, PeCB at the soil matrix significant concentrations of these contaminants could not be observed in the plume. MCB and DCB were transported much further than higher chlorinated benzenes (1).

Under strong reducing conditions microbial reductive dehalogenation presumably leads to transformation of higher chlorinated benzenes to lower CBs associated with an accumulation of MCB. This assumption is supported by groundwater samples retrieved inside the containment, which already showed different characteristics compared to DNAPL (Fig. S4). Groundwater samples from the containment (K1-K4) are dominated by DCB, MCB and lower amounts of TCB. Compared to the DCB pattern in the DNAPL 1,2-DCB is already significantly reduced in concentration suggesting preferential dehalogenation of 1,2-DCB in areas close to the DNAPL. If we further compare the groundwater obtained from inside the containment with samples retrieved from the plume (A) it can be discovered that the contaminant pattern basically is similar: high abundance of DCB and MCB. In more detail, the most abundant contaminants in the plume are MCB followed by 1,4-, 1,3- and 1,2-DCB indicating pref-

erential degradation of 1,2- and 1,3 over 1,4-DCB and an accumulation of especially MCB (Fig. S4). Inside the plume, during last 10 years, TCB only occurred close to the containment in concentration up to 190 µg L^{-1} (well Q), whereas TCB concentration in all other wells was always below 5 µg L^{-1}. Higher CBs were not detected in significant concentrations.

To provide one line of evidence for in situ biodegradation of chlorinated benzenes in the plume, stable isotope fractionation analysis (SIFA) was applied. Due to difficulties associated with the sequential degradation of chlorinated benzenes the Rayleigh approach could not be used and instead an isotope balance was calculated. The isotope values of total chlorobenzenes from inside the containment (K) and well A outside were similar (-27.6 ± 0.3 and -27.2 ± 0.2 respectively, see Fig.1 of the publication) and therefore the area around well A was defined as the source of the plume which furthers reflects also highest contaminant concentrations.

274 **Tab. S4: Carbon isotope signatures [‰] for MCB and DCB isomers for samples obtained during sampling in 2005**

	MCB			1,2-DCB			1,3-DCB			1,4-DCB		
well	C_{MCB} [µg L^{-1}]	$d^{13}C_{MCB}$ [‰]	sdev [‰]	$C_{1,2\text{-DCB}}$ [µg L^{-1}]	$\delta^{13}C_{1,2\text{-DCB}}$ [‰]	sdev [‰]	$C_{1,3\text{-DCB}}$ [µg L^{-1}]	$\delta^{13}C_{1,3\text{-DCB}}$ [‰]	sdev [‰]	$C_{1,4\text{-DCB}}$ [µg L^{-1}]	$\delta^{13}C_{1,4\text{-DCB}}$ [‰]	sdev [‰]
A*	1400	-29.6	0.13	22	-17.7	1.01	450	-23.5	0.63	970	-24.7	0.46
B*	630	-29.0	0.22	18	-15.6	1.57	150	-22.3	0.52	280	-24.6	0.19
C*	590	-27.8	0.27	29	-20.8	0.51	95	-24.8	0.27	140	-23.6	0.59
D*	560	-28.3	0.15	13	-17.0	0.26	120	-22.7	1.24	250	-24.0	0.29
E*	430	-26.4	0.32	nd	nd	nd	0.47	nd	nd	2.5	-22.4	0.06
F*	170	-27.2	0.14	0.3	nd	nd	2.1	nd	nd	10	-22.0	0.49
G*	120	-28.9	0.23	17	-22.2	0.23	82	-25.1	0.65	90	-25.2	0.36
H*	120	-26.3	0.24	nd	nd	nd	nd	nd	nd	nd	nd	nd
I*	120	-27.1	0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd
J*	86	-25.3	0.27	0.6	nd	nd	1.6	nd	nd	6.7	-24.3	0.86
K	85	-28.8	0.02	7.8	-25.5	0.11	48	-27.3	0.29	270	-27.3	0.41
L*	72	-26.9	0.09	0.6	nd	nd	2.3	-25.1	0.61	7.9	-25.2	0.41
M*	56	-25.6	0.10	nd	nd	nd	5.4	-28.1	0.29	36	-28.1	0.34
N*	42	-28.1	0.27	7.4	-20.8	0.92	44	-24.1	0.27	60	-24.5	0.15
O*	7.5	-28.4	0.22	1.4	-24.5	(n=1)	36	-27	0.16	61	-25.1	0.16
P	7.3	nd	nd	1.6	nd	nd	18	-26.5	0.43	48	-24.0	0.21
Q	1.6	nd	nd	0.6	nd	nd	7.2	-27.8	0.59	29	-25.0	0.26
R	1.6	nd	nd	nd	nd	nd	nd	nd	nd	0.11	nd	n.n.
T	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
U	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
V	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

* data used for PCA

nd not detectable

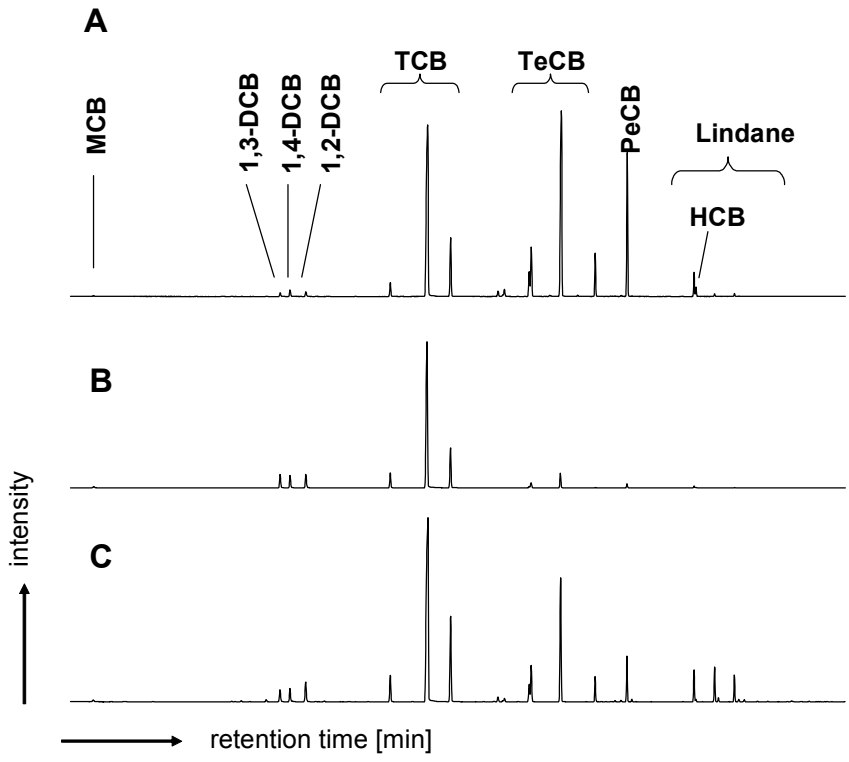


Fig. S2: Total ion chromatograms (GC-MS analysis) from three DNAPL samples (A, B, C) obtained from wells inside the containment indicating the initial composition of the contaminants. Most abundant contaminants are the higher chlorinated benzenes (TCB, TeCB, PeCB).

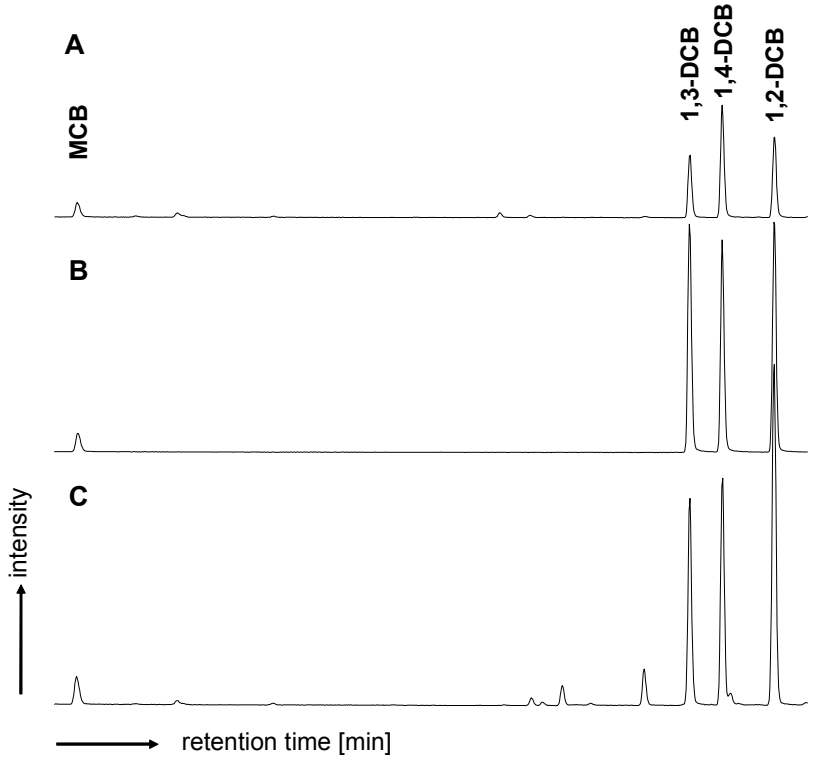
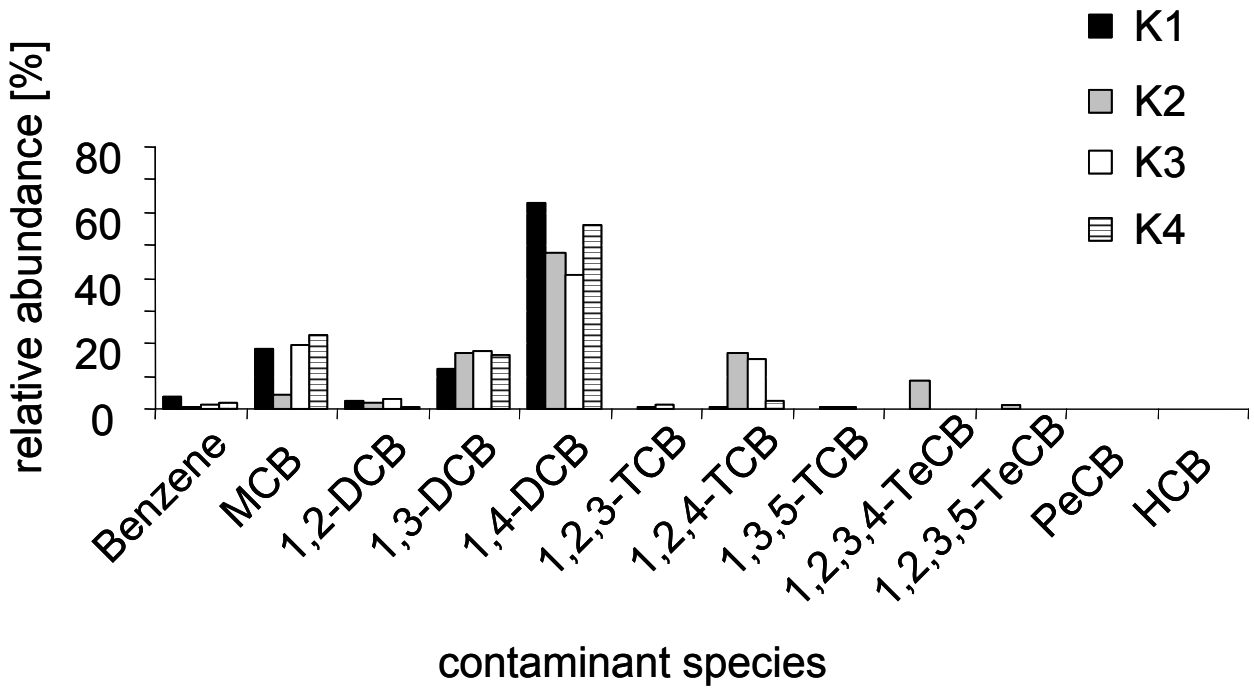


Fig. S3: Detail of the total ion chromatograms (GC-MS analysis) (see Fig. S2) from three DNAPL samples (A, B, C) obtained from wells inside the containment to better indicate the relative concentrations of MCB and DCB isomers.



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284 **Fig. S4: Relative abundance (%) of contaminants in the groundwater inside the containment (K1-K4) and in the centre**
285 **of the plume (A). As indicated by all samples in contrast to the DNAPL, dominated by TCB, TeCB, PeCB, in the**
286 **groundwater most abundant contaminant species are DCBs, MCB and TCB presumably due reductive dehalogenation**
287 **of the precursors.**

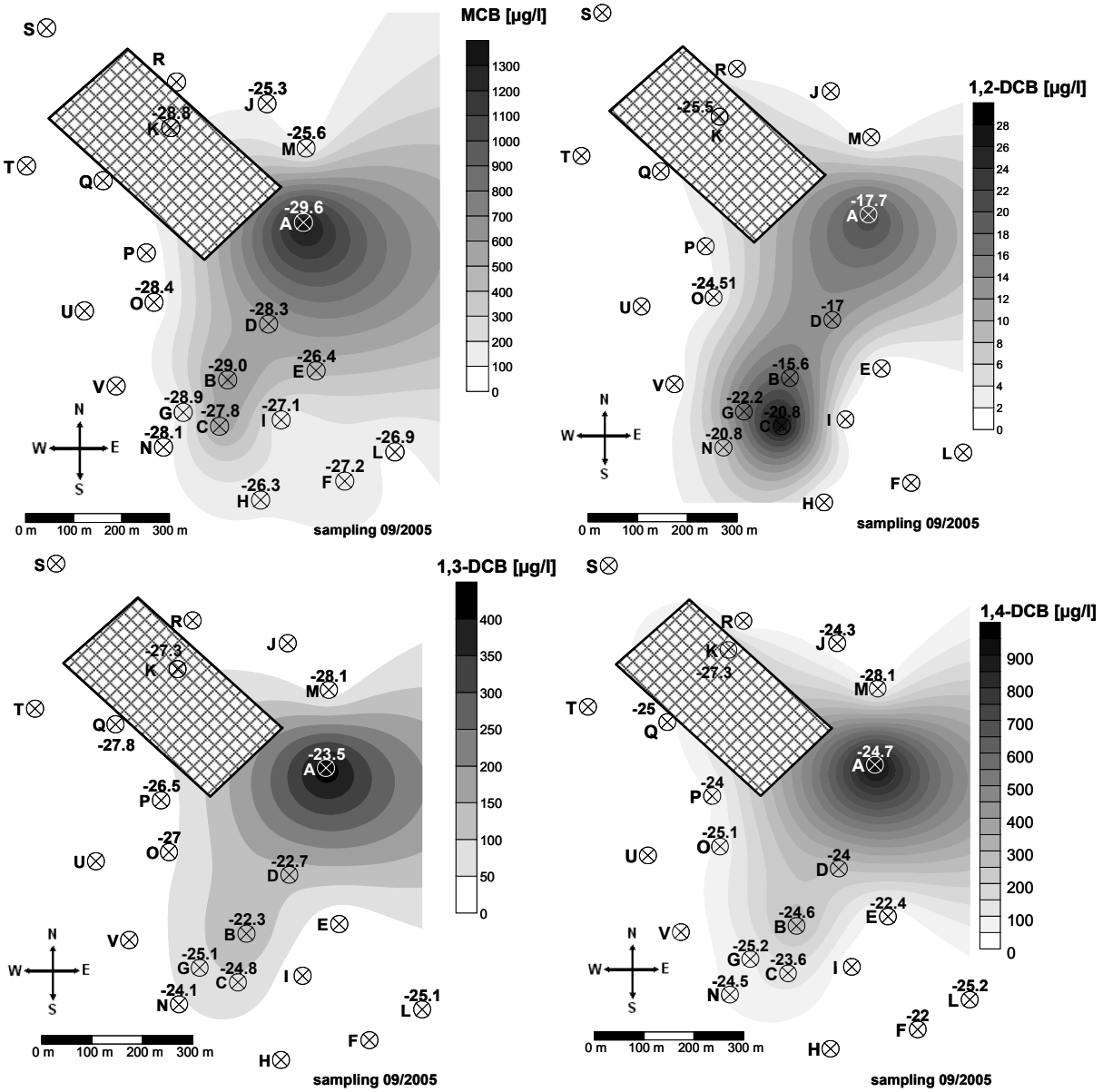


Fig. S5: Contour plots showing the concentration [µg L^{-1}] of MCB (upper left), 1,2-DCB (upper right), 1,3-DCB (lower left) and 1,4-DCB (lower right) determined for samples retrieved from the contaminated field site in September 2005. Numbers refer to the carbon isotope signatures of the respective chlorobenzene species [‰].

2.5 Assessment of plume stability

Principal component analysis was used to investigate if contaminant concentration and carbon isotope signature patterns varied over time (Fig. S6). Comparing the location of the objects (monitoring wells) no substantial shifts in the patterns were observed from year 2005 to year 2006 along the PC1, accounting for 61.3 % of the total variation suggesting relatively stable conditions over time. Additionally, the temporal evolution of contaminant concentration (2000-2007) and corresponding isotope signatures (2005-2007) were analyzed in more detail for wells H, F, L at the southern fringe of the plume (Fig S7-S9). The natural groundwater flow at wells F, G, L at the southern fringe is not affected from this activity and therefore they are suitable for assessing plume stability. Although the evolution of the plume was monitored at static points, the results still represent the by-passing groundwater flow, because during

one year the water moves approximately 30 m eastwards. Thus, in 7 years a by-passing groundwater flow of about 210 m was monitored, which may allow to reconstruct the spatial and temporal plume development. During February 2005 and September 2007 the isotope signatures of MCB and CB were monitored to obtain information on the Natural Attenuation properties at the southern fringe of the plume. In well H only MCB remained in the groundwater. During the investigate time the concentration varied significantly between 100 and 230 µg L^{-1} although during last three years the values stabilized to approximately 110 µg L^{-1}. The isotope signatures of MCB remained relatively stable or even showed slight enrichment over time indicating constant degradation conditions (Fig. S7). In well L both, MCB and DCBs are present and therefore isotope data of MCB and the isotope balance are shown in Fig S8. Again the concentration is significantly changing over time (approximately 50 to 100 µg L^{-1}). The isotope signatures also show minor fluctuations but a general isotopic enrichment compared to the source area of the plume for both, MCB and the total CB, indicates that the degrading capacity remains relatively constant.

In well F also both, MCB and total CBs were monitored (Fig S9). Concentration and the isotope signatures slightly fluctuate over time but the general trend of the isotopic enrichment compared to the source area of the plume suggests that microbial activity controls plume stability retarding further distribution of contaminants down gradient to the east.

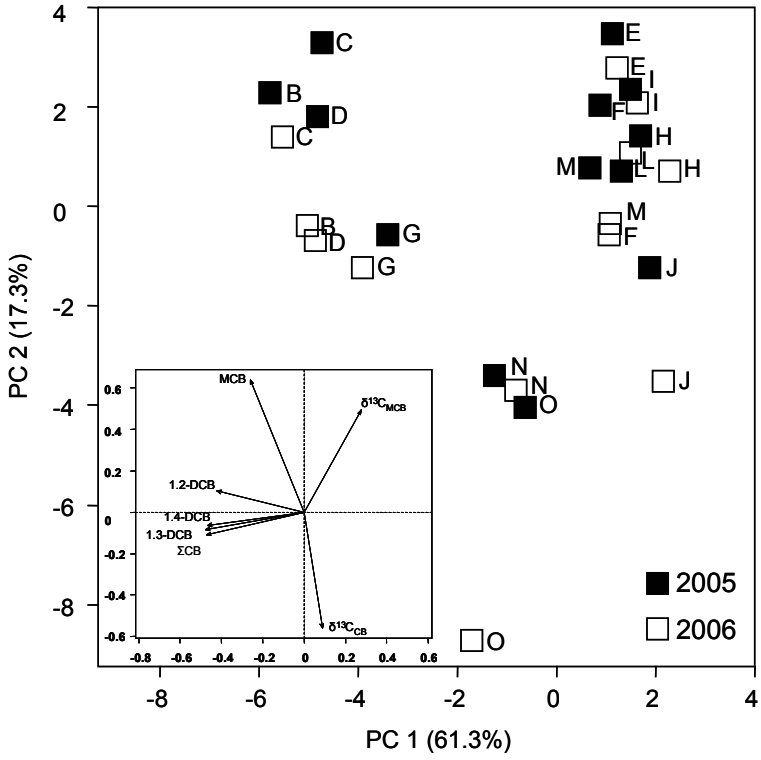
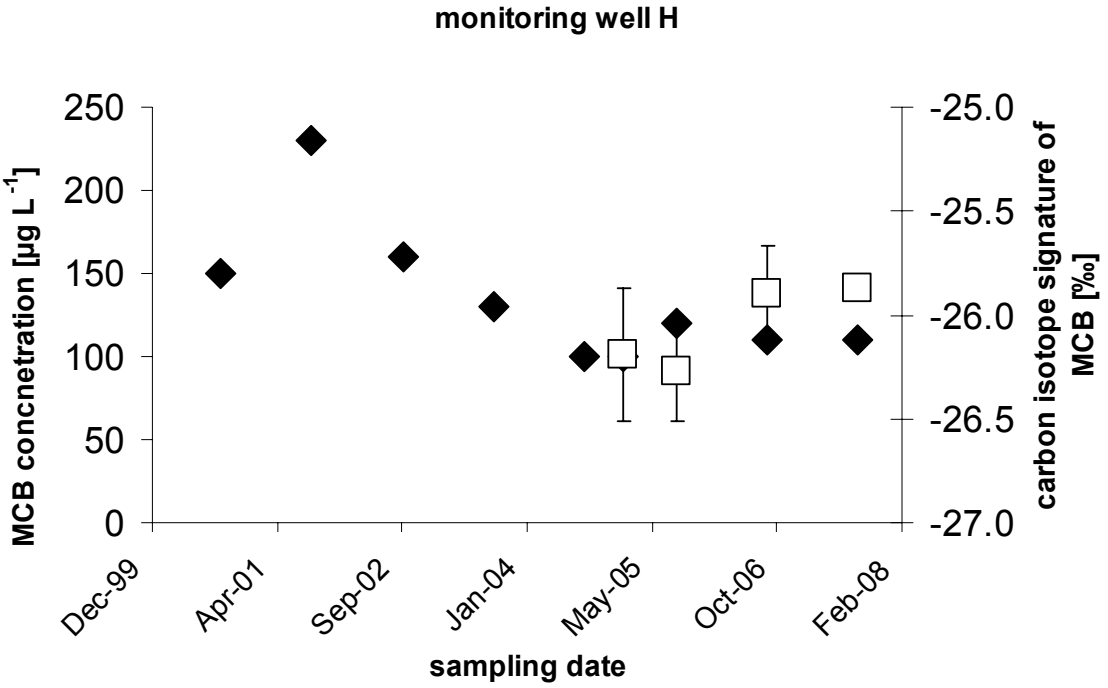
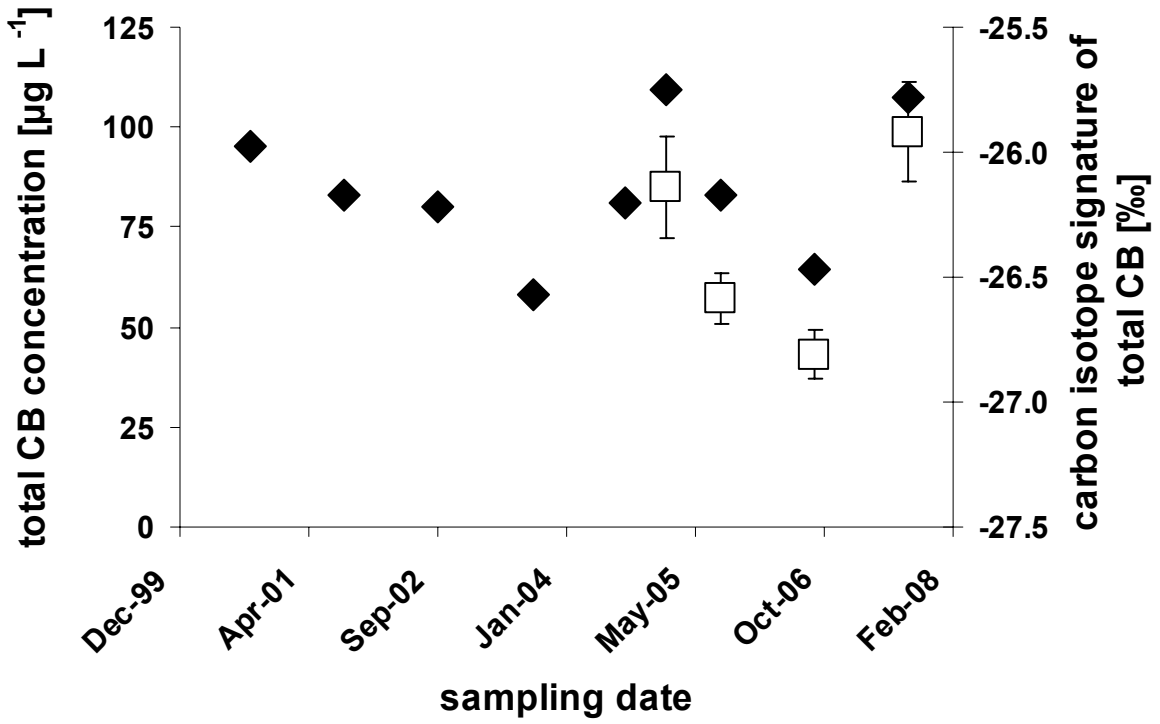


Fig. S6: PCA ordination plot of MCB, 1,2-DCB, 1,3-DCB, 1,4-DCB and total CB concentrations and carbon isotope composition in groundwater samples obtained during sampling in 09/2005 [■] and 09/2006 [□] as indicator for plume stability over time. Values on the axes indicate % of total variation explanation by the corresponding axis (PC1, principal component axis 1; PC 2, principal component axis 2). Letters indicate the different wells.



327

328 **Fig. S7: Evolution of concentration (♦) [µg L^{-1}] and carbon isotope signature (□) of MCB [‰] over time at monitoring**
329 **well H at the S-fringe of the plume to assess plume stability. At monitoring well L no DCBs are present. For compari-**
330 **son, the isotope composition of total CBs in the source area (well A) is -27.2 ± 0.2 ‰.**



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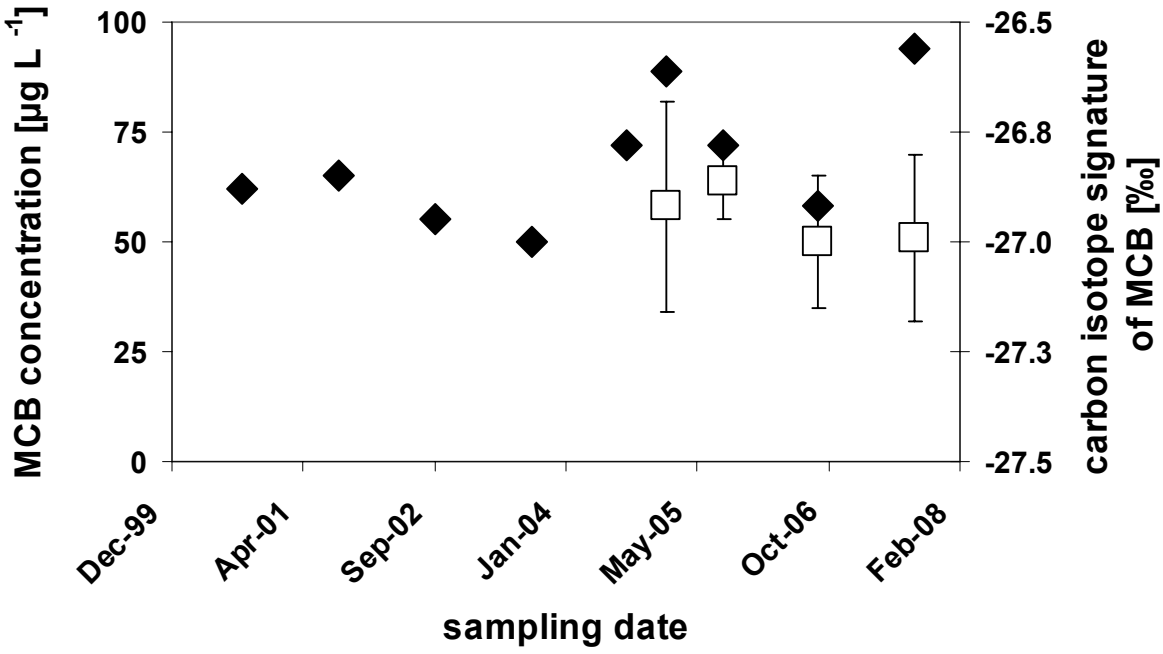
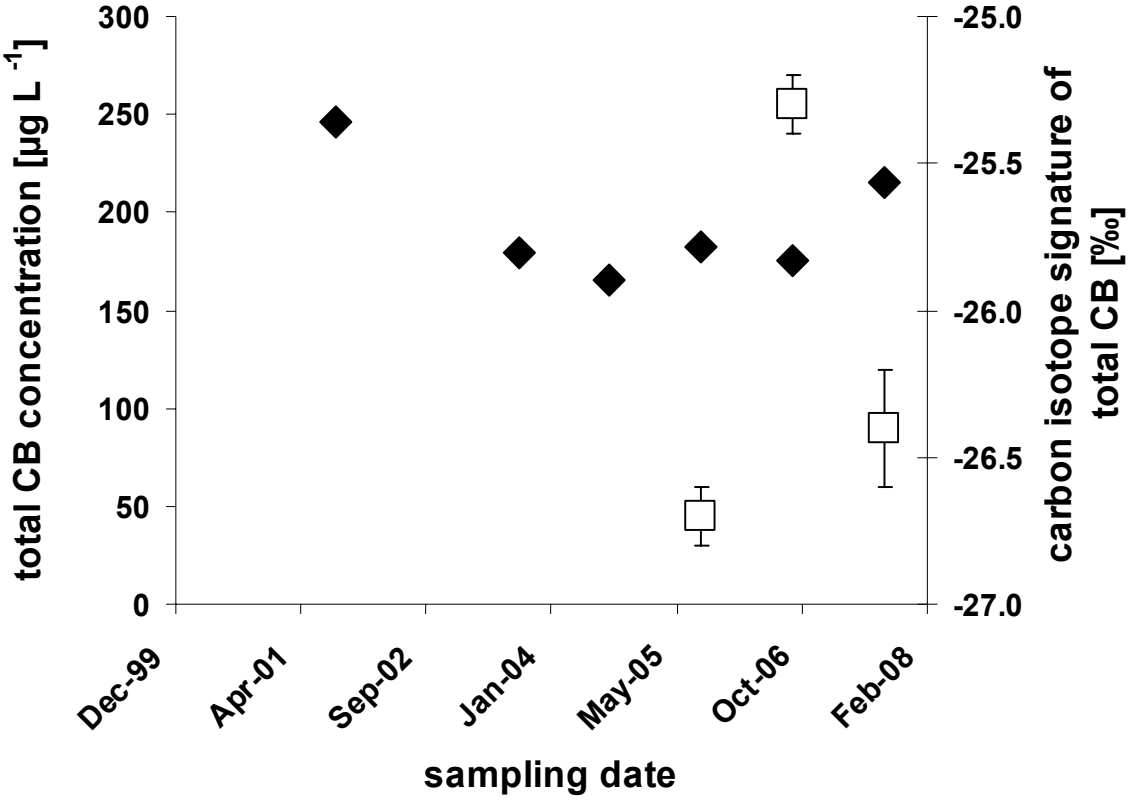


332

333 **Fig. S8: Evolution of concentration (♦) [µg L^{-1}] and carbon isotope signature (□) of MCB (lower) and total CBs (upper)**
334 **[‰] over time at monitoring well L at the SE fringe of the plume to assess plume stability. At monitoring well L DCBs**
335 **are present (10-20 %), therefore an isotope balance has to be quantified. For comparison, the isotope composition of**
336 **total CBs in the source area (well A) is -27.2 ± 0.2 ‰.**

337



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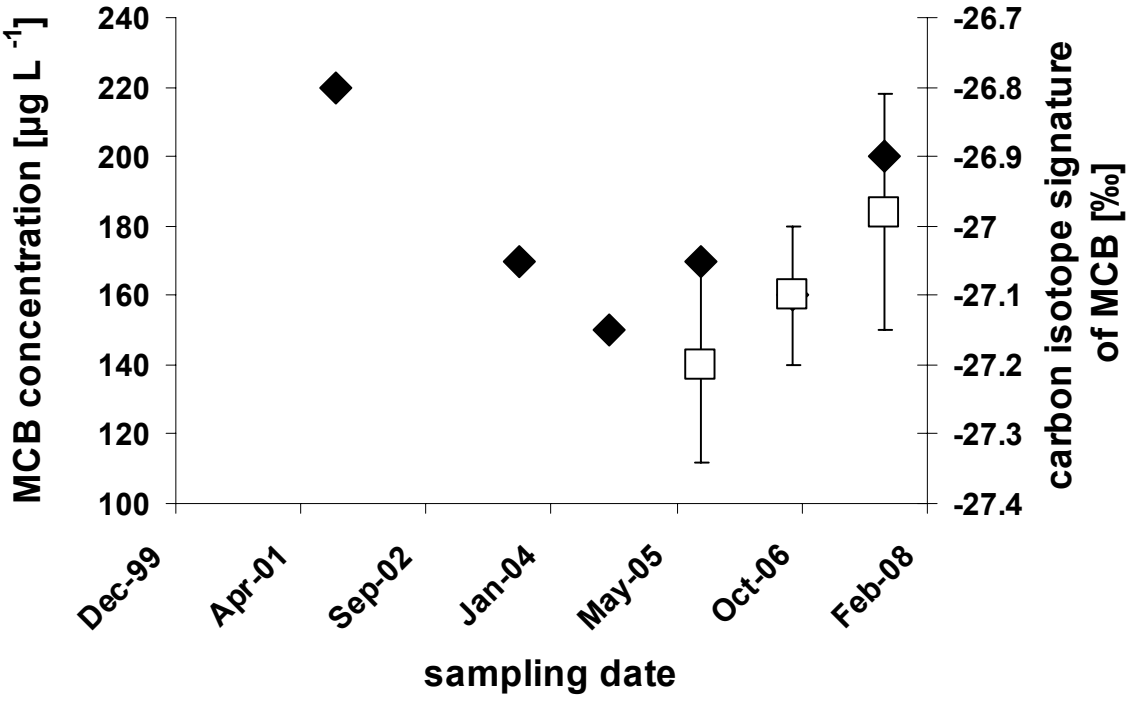

339

340 **Fig. S9: Evolution of concentration (♦) [µg L^{-1}] and carbon isotope signature (□) of MCB (lower) and total CBs (upper)**
341 **[‰] over time at monitoring well F at the SE fringe of the plume to assess plume stability. At monitoring well F DCBs**
342 **are present (8 %), therefore an isotope balance has to be quantified. For comparison, the isotope composition of total**
343 **CBs in the source area (well A) is -27.2 ± 0.2 ‰.**

344

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Anhang G

Assessment of *in situ* biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland (2007)

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Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland

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An integrated approach including isotope composition analysis and in situ microcosm experiments provided evidences for in situ biodegradation of MCB in a wetland system.

Abstract

The degradation of monochlorobenzene (MCB) was assessed in a constructed wetland treating MCB contaminated groundwater using a detailed geochemical characterisation, stable isotope composition analysis and in situ microcosm experiments. A correlation between ferrous iron mobilisation, decreasing MCB concentration and enrichment in carbon isotope composition was visible at increasing distance from the inflow point, indicating biodegradation of MCB in the wetland. Additionally, in situ microcosm systems loaded with ^{13}C-labelled MCB were deployed for the first time in sediments to investigate the biotransformation of MCB. Incorporation of ^{13}C-labelled carbon derived from the MCB into bacterial fatty acids substantiated in situ degradation of MCB. The detection of ^{13}C-labelled benzene indicated reductive dehalogenation of MCB. This integrated approach indicated the natural attenuation of the MCB in a wetland system. Further investigations are required to document and optimise the in situ biodegradation of MCB in constructed and natural wetland systems treating contaminated groundwater.

Keywords: Constructed wetland; Monochlorobenzene; Biodegradation; In situ microcosms; Isotopic fractionation

1. Introduction

Monochlorobenzene (MCB) is encountered worldwide as a groundwater pollutant, and persists in the essentially anaerobic aquifer at the large-scale contaminated site in Bitterfeld, Germany (Heidrich et al., 2004; Wycisk et al., 2004). In recent years, interest has grown in using phytoremediation processes for the elimination of recalcitrant organic substances from waste- and groundwater (Macek et al., 1998; Schnoor et al., 1995; Shimp et al., 1993; Trapp, 2000) including chloroaromatics (Gilbert and Crowley, 1997). Wetland systems represent an effective and inexpensive option to treat groundwater polluted with organic compounds by taking advantage of the geochemical and biological processes (e.g. Baker, 1998; Dunbabin and Bowmer, 1992; Gumbricht, 1993). Indeed, rapid degradation of chlorinated organics has been observed in the rhizosphere (Anderson and Walton, 1995; Jordahl et al., 1997; Lorah and Olsen, 1999; Pardue et al., 1996).

While aerobic degradation of MCB has been well studied (e.g. Van Agteren et al., 1998), only some evidence for MCB

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transformation under anoxic conditions has been presented yet and the degradation pathway is unknown (Kaschl et al., 2005; Liang and Gribic-Galic, 1990; Nowak et al., 1996). Moreover, only very few studies focus on the anaerobic microbial transformation of MCB under field conditions. Recently, indications of anaerobic MCB degradation taking place in the Bitterfeld contaminated aquifer were provided on the basis of isotope fractionation patterns (Kaschl et al., 2005). Kinetic isotope fractionation processes have been employed to demonstrate the biological transformation of various contaminants (Richnow et al., 2003a,b; Sherwood Lollar et al., 2001; Song et al., 2002). A substantial enrichment of ^{13}C in the non-degraded fraction in the course of a contaminant plume indicates microbial degradation, as dilution and sorption do not affect the isotope composition of contaminants significantly (Harrington et al., 1996; Schüth et al., 2003; Slater et al., 2000). Combining stable isotope composition analysis with information obtained from simple in situ microcosm experiments (BACTRAPs) using isotope labelled substrate may provide a suitable approach to qualitatively support in situ biotransformation and to monitor spatial and temporal natural attenuation processes. Previously, BACTRAPs were exclusively installed in groundwater monitoring wells (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006) and were deployed in sediment for the first time in the framework of this study.

For the assessment of in situ biodegradation in constructed wetlands and wetlands treating contaminated groundwater, it may be necessary to use several methods providing more than one line of evidence. A combined approach may be of additional benefit in particular when systems are complex, possess several compartments and convincing evidence is required. Moreover, a better understanding of the controlling geochemical processes in wetland systems is necessary to reliably predict the retention and transformation of contaminant. In this study, we evaluated the natural attenuation of MCB in a constructed wetland treating MCB contaminated groundwater using a detailed geochemical characterisation, stable isotope composition analysis and in situ microcosm experiments. The spatial variations of geochemical parameter were studied with the help of multivariate statistics to investigate the main processes controlling the wetland system. The concentration and carbon stable isotope composition of MCB was analysed to monitor the in situ contaminant degradation and in situ microcosms were used to provide qualitative evidences of in situ biotransformation of MCB.

2. Materials and methods

2.1. Design and characteristics of the wetland

The pilot scale constructed wetland at the experimental site in Bitterfeld was set up in December 2002. The horizontal subsurface flow wetland consisted of a stainless steel tank divided into two segments. Each segment was 6 m × 1 m and was filled to an average depth of 0.5 m with autochthonous quaternary aquifer material consisting predominantly of Bitterfeld mica sand (25%) and gravel (67%), which was embedded in lignite (10%) with an effective porosity of 28% (Vogt et al., 2002). The hydrogeochemical characteristics of the study site and the filling material originating from the local aquifer are described in previous studies (e.g. Vogt et al., 2002; Weiss et al., 2001). One of the segments was planted with common reed (*Phragmites australis*, Cav.), whereas the other side was left unplanted. In both segments, a 1 m long open water pond at the outflow side allows direct contact between the atmosphere and the water surface. The water level was maintained at approximately 10 cm below the surface of the wetland. The groundwater was collected from the MCB contaminated aquifer and conveyed from 16 to 22 m depth directly to the wetland. Both segements were operated in a flow-through mode at a flow rate of 4.7 L h^{-1}, corresponding to a retention time of 6 days.

2.2. Sampling

In the period from April to September 2005, pore water samples were collected five times (d0, d53, d66, d143, d172) in order to investigate the geochemical processes and the contaminant behaviour in the wetland system. The pore water was collected in both segments along a transect from the inflow up to the outflow of the wetland, at respectively 0 (inflow valves), 0.5, 1, 2, 3 and 4 m using a stainless steel lance. At each sampling point, three depths, 30, 40 and 50 cm were systematically investigated. Water samples were also collected at the ponds (6 m).

In addition, to assess the in situ biodegradation using isotope composition analysis, pore water samples from both segments were collected at day 0 and day 53, at 0, 1 and 3.5 m along the wetland at 0.5 m depth, as well as in the ponds.

2.3. Physico-chemical and geochemical parameters of the pore water samples

The redox potential was measured on-line in the field using a SenTix ORP electrode (PT 1000, PreSens, Regensburg, Germany). The temperature was determined by a temperature sensor (PT 1000, PreSens, Regensburg, Germany). Samples for the pH analysis and the quantitative ions were filtered through a 5-μm syringe filter (Ministart NML, Sartorius) for particle removal. The pH value was measured with a SenTix41 electrode with pH 537 Microprocessor (WTW, Weilheim, Germany). Oxygen measurement was carried out using an optical oxygen trace sensor system (oxygen meter Fibox-3-trace and flow-through cell type sensor FTC-TOS7) with automatic temperature compensation (temperature sensor PT 1000) (PreSens, Regensburg, Germany). For the analysis of Mn(II), total iron and Fe(II), hydrochloric acid was added and samples were diluted with deionised water (1:10, v:v). Total iron and Mn(II) concentrations were analysed by atomic emission spectrometry with ICP excitation and CCD detection (Spectro Ciros Vision CCD, Spectro Analytical Instruments, Kleve, Germany). Photometric analysis of ferrous iron was carried out at 562 nm after derivatisation with ferrocin using a Cadas 100 photometer (Hach Lange, Düsseldorf, Germany). Chloride and sulphate concentrations were determined by ion chromatography (DX 500) with conductivity detection (CD 20) and an IonPacAG11 (4 × 250 mm) column (Dionex Corporation, Sunnyvale, USA). For the analysis of sulphide concentrations samples were spiked with sulphide anti-oxidant buffer (200 ml L^{-1} 10 M NaOH, 35 g L^{-1} ascorbic acid, 67 g L^{-1} EDTA) (1:1, v:v) and measured with an ion selective Ag/S 500 electrode and reference electrode R 503 (WTW, Weilheim, Germany).

2.4. Analysis of benzene, MCB and metabolites

Pore water samples for the analysis of benzene and MCB concentrations were collected in 20 mL glass flasks (Supelco, Bellefonte, USA), and sealed with Teflon-lined septa. Sodium azide solution was added to the samples to inhibit microbial activity. Benzene and MCB concentrations were quantified by automatic headspace gas chromatography using an HP 6890 gas chromatograph with flame ionisation detector (Agilent Technologies, Palo Alto, USA). For headspace analysis a volume of 1000 μl was injected at an injection temperature of 250 °C with split 1:5 (measurements in duplicates). The chromatographic separation was achieved on an HP-1 capillary column (Agilent Technologies, Palo Alto, USA) (30 m × 0.32 mm × 5 μm) with the following oven temperature program: 45 °C (1 min), 20 °C min^{-1} to 200 °C (2.5 min), 65 °C min^{-1} to 250 °C (1 min) and a detector temperature of 280 °C.

For the determination of the carbon isotope composition of MCB, 1 L glass bottles (Schott, Mainz, Germany) containing NaOH pellets to prevent

microbial growth were filled completely with groundwater, stored at 4 °C and extracted within 24 h using 2 mL *n*-pentane as described previously (Richnow et al., 2003b). The analysis of volatile metabolites obtained in the in situ microcosm experiments was carried out using an HP 6890 gas chromatograph with HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, USA). Aliquots of 1 μl liquid samples were injected at a temperature of 280 °C with split 1:40 and separated on a Zebron BPX-5 column (30 m × 0.32 mm × 0.25 μm) (Phenomenex, Torrance, USA). The following oven temperature program was applied: 40 °C (2.5 min), 10 °C min^{-1} to 70 °C (0 min), 60 °C min^{-1} to 280 °C (4 min).

2.5. In situ microcosms (BACTRAPs)

2.5.1. Preparation of microcosms and derivatisation of fatty acids

The in situ microcosms were prepared as described previously (Stelzer et al., 2006). Different sets of in situ microcosm experiments were prepared. One set was loaded with [$^{13}C_6$]-labelled MCB (Cambridge Isotope Laboratories, Andover, USA) and another one with natural abundance MCB. A third set was kept unloaded to observe the background effects. The loading was done via gas phase under reduced pressure with approximately 40 mg MCB per g Bio-Sep®. The microcosms were deployed at 1.5, 2.5 and 4.5 m from the inflow in both planted and unplanted segments at 50 cm depth. The microcosms were collected after 6 weeks and fatty acid extraction was carried out according to Bligh and Dyer (1959). The derivatisation to obtain fatty acid methyl esters (FAME) was done according to Thiel et al. (2001). After evaporation to complete dryness and addition of heneicosanoic acid methyl ester (C21:0) as an internal standard the FAME fraction was dissolved in *n*-hexane for further identification, structural characterisation and carbon isotope composition analysis.

2.5.2. GC–MS analysis

For identification and structural characterisation of FAME, an HP 6890 gas chromatograph coupled with an HP 5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, USA) was used. The FAME were separated on a Zebron BPX-5 column (30 m × 0.32 mm × 0.25 μm) (Phenomenex, Torrance, USA) with the following temperature program: 70 °C (1 min), 20 °C min^{-1} to 130 °C, 2 °C min^{-1} to 150 °C (5 min), 2 °C min^{-1} to 165 °C (5 min), 2 °C min^{-1} to 230 °C, 20 °C min^{-1} to 300 °C (5 min). FAME were identified by comparing with the retention time and mass spectra of an authentic standard mix (bacterial acid methyl esters mix, Sigma–Aldrich, Germany) and quantified relatively to the internal standard.

2.6. Isotopic composition analysis

The carbon isotope composition of MCB and the FAME was measured with a gas chromatography–combustion–isotope ratio mass spectrometry system (GC–C–IRMS) consisting of a GC unit (HP 6890, Agilent Technologies, Palo Alto, USA), a combustion device (Finnigan MAT GC III, ThermoFinnigan Bremen Germany) with water-removal assembly (Nafion® membrane, 50 cm long, $T = 0$ °C) and a mass spectrometer (Finnigan MAT 252; ThermoFinnigan, Bremen, Germany), as previously described (Richnow et al., 2003a). Helium was used as carrier gas at a flow rate of 1.5 ml min^{-1}.

Stable isotope samples were measured in triplicates and the analyses were carried out immediately after each sampling. Aliquots of 1 μl liquid samples were injected at 250 °C with split 1:10 to the GC–C–IRMS and separated on a capillary column (Zebron ZB-1, 60 m × 0.32 m × 1 μm; Phenomenex, Torrance, USA). The following chromatographic conditions were applied: injector temperature 250 °C, oven temperature program: 40 °C (1 min), 4 °C min^{-1} to 150 °C, 20 °C min^{-1} to 250 °C (2 min). The carbon isotope composition is reported in the delta notation as $\delta^{13}C$ values [‰] relative to Vienna Pee Dee Belemnite Standard (V-PDB, IAEA-Vienna) (Eq. 1) (Hoefs, 1997).

$$\delta^{13}C[‰] = \left(\frac{(^{13}C/^{12}C)_{Sample}}{(^{13}C/^{12}C)_{Standard}} - 1\right) \times 1000 = \left(\frac{R_{Sample}}{R_{Standard}} - 1\right) \times 1000 \quad (1)$$

Eq. (2) is applied to calculate the remaining substrate fraction (f_t) using the isotope fractionation factor (αC).

$$f_t = \frac{R_t}{R_0} \exp\left(\frac{1}{1/\alpha C - 1}\right) \quad (2)$$

The R_t and R_0 give the isotope composition of MCB at time t and zero. Eq. (3) is applied to calculate the percentage of biodegradation of the residual substrate fraction (B_t).

$$B[\%] = (1 - f_t) \times 100 \quad (3)$$

For the separation of the FAME fractions, a Restek RTX 5 column (60 m × 0.32 mm × 0.1 μm; Restek, Bellefonte, USA) was used with the same temperature program applied for the GC–MS-analysis of FAME. Aliquots of 1 μl were injected with split 1:5. The methylation of fatty acids for gas chromatographic analysis introduces an additional carbon atom into the structure of the fatty acid molecules which affects its isotopic composition. Therefore the isotope signature of fatty acids ($\delta^{13}C_{FA}$) was corrected for the isotope effect upon derivatisation to FAME with methanol as described previously (Abraham et al., 1998; Abrajano et al., 1994; Goodmann and Brenna, 1992). The methanol used for the derivatisation had an isotope composition of −38.2‰.

2.7. Statistical analysis

Statistical analyses were carried out using the R Software (R, Version 2.1.1, 2005). Statistical significance of the difference in geochemical parameters as well as concentrations and isotopic compositions of MCB between the planted and unplanted segment was determined with the unpaired Wilcoxon or the Kruskal–Wallis rank sum tests. Correlation analyses were carried out using the Spearman rank sum coefficient. Principal component analysis (PCA) was used to analyse the relationship between the different samples with reference to their respective pore water parameters. The sampling location corresponds to the object and the chemical parameters to the descriptors (represented by the vectors) of the multivariate analysis. The PCA were scaled as correlation biplots.

3. Results

3.1. Characterisation of pore water chemistry

3.1.1. Distribution of MCB and benzene

The MCB concentration was measured as a function of the distance from the inflow point in both the planted and unplanted segment (Table 1). The average amount of MCB ranged from 14.4–17.7 mg L^{-1} at the inflow down to 2.0–2.2 mg L^{-1} in the ponds for the planted and the unplanted segment, respectively. No significant difference in MCB concentration among the three depths over the study period was generally observed ($p < 0.05$). Benzene was found in low concentration in both segments (<26 μg L^{-1}), with generally higher concentration values in the unplanted segment (Table 1).

3.1.2. Pore water geochemistry

The evolution of oxygen, redox potential, manganese, sulphide, sulphate, ferrous iron and total iron were monitored along the flow path at three depths in order to characterise the geochemical conditions prevailing in the wetland system. The average values of the three depths investigated at each sampling point (0, 0.5, 1, 2, 3, 4 m from the inflow) were computed (Table 1). To investigate the differences between the sampling locations and to explore existing gradients, the data sets were analysed by principle component analysis, separately for the unplanted and the planted segments (Fig. 1). In both cases, the vectors representing the temperature, Fe(II), and total

Table 1
Geochemical characterisation, MCB and benzene concentrations of samples collected from (a) unplanted segment and (b) planted segment

Sampling point [m from inflow]	pH	Temp. (°C)	O_2 [mg L^{-1}]	Eh [mV]	Fe(II) [mg L^{-1}]	Sulphate [mg L^{-1}]	Cr [mg L^{-1}]	Benzene [μg L^{-1}]	MCB [mg L^{-1}]
(a)									
0	7.1 (3.6)[d]	14.8 (8.5)[d]	0.04 (0.02)[e]	38 (37)[d]	0.18 (0.23)[c]	968.6 (401.81)[c]	280.4 (116.26)[c]	25.6 (3.6)[c]	17.73 (7.98)[c]
0.5	7.0 (2.2)[b]	16.3 (5.4)[a]	0.05 (0.04)[b]	61 (26)[b]	2.43 (1.83)[a]	969.5 (253.35)[a]	272.8 (68.93)[a]	21.2 (6.1)[a]	14.58 (5.04)[a]
1	6.7 (2.1)[a]	17.1 (5.4)[a]	0.04 (0.02)[a]	84 (38)[a]	9.58 (5.80)[a]	966.6 (252.74)[a]	269.3 (67.63)[a]	23.3 (3.9)[a]	14.50 (5.11)[a]
2	6.9 (2.0)[a]	17.8 (5.5)[a]	0.04 (0.04)[a]	70 (41)[a]	12.40 (6.74)[a]	973.2 (253.79)[a]	267.2 (67.55)[a]	22.2 (5.4)[a]	14.47 (5.23)[a]
3	6.8 (1.9)[a]	18.4 (5.5)[a]	0.04 (0.03)[a]	57 (34)[a]	21.12 (7.45)[a]	974.4 (254.08)[a]	261.2 (71.13)[a]	22.5 (5.5)[a]	14.95 (5.56)[a]
4	6.8 (1.9)[a]	18.9 (5.6)[a]	0.04 (0.02)[a]	57 (37)[a]	30.23 (7.08)[a]	973.2 (256.05)[a]	267.3 (72.04)[a]	21.1 (6.3)[a]	14.43 (5.53)[a]
Pond	6.8 (3.0)[d]	20.9 (8.4)[d]	n.a.	250 (44)[d]	3.06 (8.81)[c]	985.7 (402.98)[c]	287.3 (117.57)[c]	1.8 (3.0)[c]	2.01 (7.90)[c]
(b)									
0	6.8 (3.07)[d]	14.83 (8.47)[d]	0.04 (0.02)[e]	35 (40)[d]	0.20 (0.21)[c]	977.0 (399.84)[c]	261.8 (109.16)[c]	24.0 (6.9)[c]	14.44 (6.42)[c]
0.5	6.8 (2.18)[b]	15.58 (5.46)[a]	0.13 (0.05)[b]	47 (42)[b]	5.73 (3.62)[a]	994.2 (250.73)[a]	257.3 (68.50)[a]	11.8 (5.5)[a]	8.71 (7.67)[a]
1	6.8 (2.07)[a]	16.63 (5.39)[a]	0.12 (0.09)[a]	54 (40)[a]	13.34 (7.48)[a]	979.7 (247.82)[a]	256.4 (67.76)[a]	15.7 (6.6)[a]	9.78 (7.25)[a]
2	6.7 (1.97)[a]	17.64 (5.37)[a]	0.05 (0.02)[a]	46 (36)[a]	25.48 (7.60)[a]	1006.2 (248.04)[a]	266.3 (68.18)[a]	11.9 (8.8)[a]	8.20 (6.57)[a]
3	6.7 (1.90)[a]	18.02 (5.43)[a]	0.05 (0.03)[a]	51 (34)[a]	36.57 (7.33)[a]	999.7 (248.04)[a]	271.1 (69.01)[a]	11.3 (8.9)[a]	7.94 (6.07)[a]
4	6.5 (1.92)[a]	18.82 (5.55)[a]	0.03 (0.02)[a]	57 (34)[a]	37.40 (8.94)[a]	998.4 (259.16)[a]	265.8 (73.69)[a]	10.2 (8.0)[a]	7.73 (5.86)[a]
Pond	6.8 (3.12)[d]	20.53 (8.06)[d]	4.85 (1.89)[e]	204 (43)[d]	2.42 (9.28)[c]	1068.2 (397.40)[c]	274.8 (110.69)[c]	0.2 (0.4)[c]	2.20 (7.77)[c]

Values represent the depth profiles average in the soil compartments (0.5–4 m) and average (0 m and pond) over the study period (13.04.05 to 29.09.05). Standard deviation is indicated in parentheses. n.a., Not assessed.

[a] Profile average of five sampling dates ($n = 15$).

[b] Profile average of four sampling dates ($n = 12$).

[c] Average of five sampling dates ($n = 5$).

[d] Average of four sampling dates ($n = 4$).

[e] Average of three sampling dates ($n = 3$).

Fe parameters were orientated in the same direction (positive correlation), in the opposite direction with regards to vectors representing manganese and ammonium (negative correlation), and are perpendicular to the MCB vector (absence of correlation). The orthogonal projection of an object on a descriptor allows approximating the correlation between that object and the descriptor. For both segments, samples from the inflow part of the wetland (0.5–1 m) were associated with MCB, manganese and ammonium, whereas samples from the outflow (3 and 4 m) were generally associated with total Fe and Fe(II). Indeed, the Fe(II) concentration at the inflow was below 0.5 mg L^{-1} and systematically increased along the flow path in both planted and unplanted segment, indicating ferrous iron mobilisation. A clear shift in the parameters characterising predominantly the trends of variation of the samples along the flow path is operating on the first principal component. Indeed, the axis I corresponds to a spatial gradient from the inflow to the outflow of the system, and separates sampling sites accordingly (from the right to the left). The variations of geochemistry along the flow path contributed more in characterising the samples than the variations occurring along the vertical profile.

The maximum concentration of Fe(II) reached an average value of 37.4 mg L^{-1} at 4 m from the inflow of the planted segment. The levels of total dissolved iron detected throughout the experimental period ranged from 0.8 to 50.6 mg L^{-1}. Interestingly, Fe(II) mobilisation was more important in the planted than in the unplanted segment. Correlation analysis revealed, however, very similar Fe(II) mobilisation patterns in both segments over the study period (Spearman's rank correlation coefficient, $\rho > 0.9$; $p < 0.01$). Iron reduction was apparently a relevant process at the three depths. Due to the high background concentration of sulphate in the supplied groundwater (700–1100 mg L^{-1}), a sensitive analysis of the microbial sulphate reduction on the basis of sulphate concentration was not possible. However, trace concentrations of sulphide (<3 μg L^{-1}) were detected along the horizontal transect, indicating sulphate reduction. Mn(II) concentrations systematically ranged below 0.2 mg L^{-1}, suggesting a low relevance of Mn as electron acceptor in the wetland (data not shown). The redox potential displayed averages of values ranging from 35 mV at the inflow to 250 mV in the ponds, and maximal concentration of oxygen in the soil compartments was 0.12 mg L^{-1}, indicating the prevalence of anoxic conditions in the soil compartments and oxic conditions in the ponds, respectively. The dissolved organic and total carbon concentrations ranged from 0.24 to 0.45 mM L^{-1} and from 2.27 to 2.41 mM L^{-1} in the unplanted and planted segments, respectively (data not shown). Nitrate concentrations were assessed in previous studies and ranged systematically under the detection threshold in the groundwater supplied to the wetland (Vogt et al., 2002), and nitrate may therefore not represent a relevant electron acceptor. The chloride concentrations did not show any significant variation along the transect.

3.2. Carbon isotopic composition of MCB

The concentration and isotopic composition ($\delta^{13}C$) of MCB were plotted as a function of the distance from the inflow (Fig. 2). A decrease in MCB concentrations over the flow path was systematically associated in both segments with a significant enrichment in $\delta^{13}C$. In the planted segment, MCB showed a maximal isotope shift of 0.6‰, whereas it reached 0.9‰ in the unplanted segment. This indicates that MCB is subjected to in situ biodegradation in both segments.

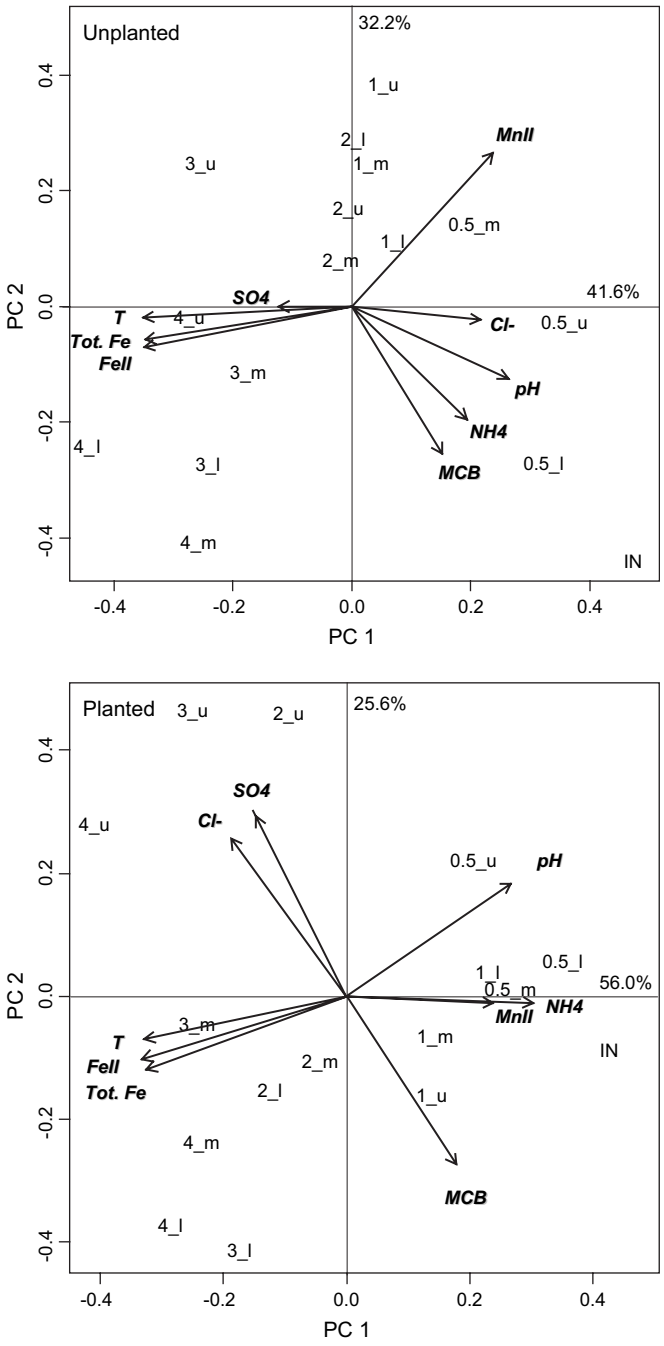

Fig. 1. Ordination plot generated by principal component analysis representing the relationship between the sampling locations and the average geochemical parameters measured in both the planted (a) and the unplanted (b) experimental wetlands over the study period. Description vectors correspond to: FeII, ferrous iron; Tot.Fe, total iron (Fe(II) + Fe(III)); MCB, monochlorobenzene; MnII, manganese(II); NH4, ammonium; T, temperature. Objects correspond to: 0.5–4, distance [m] from the inflow point; u, upper depth (30 cm); m, medium depth (40 cm); l, lower depth (50 cm). Values on the axes indicate % of total variation explained by the axes (PC 1, principal component axis 1; PC 2, principal component axis 2).

Assuming a isotopically homogenous source of MCB, these values are slightly higher than the typically defined analytical error of 0.5‰ associated with compound specific isotope analysis (Dempster et al., 1997; Mancini et al., 2002).

A quantitative assessment of in situ MCB biodegradation requires a fractionation factor representing the in situ conditions. Fractionation factors for aerobic MCB degradation are available (Kaschl et al., 2005), whereas factors retrieved under laboratory conditions for anaerobic MCB degradation are missing. Although MCB degradation under anaerobic conditions is expected in the wetland, Eq. (3) allows estimating the significance of degradation along the flow path throughout the wetland applying the highest isotope fractionation factors (αC) retrieved for aerobic MCB degradation by a dioxygenase reaction pathway ($\alpha C = 1.0004$) (Kaschl et al., 2005). The quantification was carried out based on the isotope signature of MCB measured at the planted side at day 0. The lowest $\delta^{13}C$ value measured (inflow point, 0 m: MCB $\delta^{13}C = -27.0‰$) was used as the initial isotope composition of the source (R_0). The estimated percentage of biodegradation was 60% of the inflowing MCB mass at 3.5 m, whereas the observed contaminant mass decrease reached only 38% at 4 m from the inflow. Processing of isotope and concentration data of the second sampling campaign gave almost identical results.

The use of too low fractionation factors for the quantification of MCB degradation would lead to an overestimation of the effective MCB mass depletion at the planted segment. The observed absence of significant MCB mass depletions along with a higher isotopic shift at the unplanted segment obviously direct to the same conclusion. Therefore, higher fractionation factors are expected, which would be more in concordance with the observed MCB concentration values. The use of higher isotope fractionation factors would point to a dominating anaerobic fractionation process, which is compatible with the observation of anoxic conditions in the wetland. The fractionation factors would then be comparable to the ones retrieved by Kaschl et al. (2005) ($\alpha C = 1.0005$) in the local anaerobic aquifer with MCB contamination or by Griebler et al. (2004) and Mancini et al. (2003), obtaining significantly higher fractionation factors for the anaerobic degradation of benzene or trichlorobenzenes.

3.3. In situ microcosm experiment

[$^{13}C_6$]-labelled MCB was used as stable isotope tracer in the in situ microcosm experiment in the wetland. The BACTRAPs were incubated directly in both, soil compartments, at several distances from the inflow, as well as in the ponds. The incorporation of labelled carbon derived from the labelled substrate into bacterial fatty acids provided evidence for MCB degradation in the wetland system. In addition, [$^{13}C_6$]-benzene, a possible intermediate, found on the BACTRAPs from both segments demonstrated the occurrence of reductive dehalogenation of MCB (Fig. 3). Even though the employed [$^{13}C_6$]-MCB contained 0.02% of [$^{13}C_6$]-benzene as impurity, significantly higher amounts of [$^{13}C_6$]-benzene were detected by GC–MS.

3.3.1. Fatty acid composition

The composition of total fatty acid fractions extracted from in situ microcosms was compared to investigate variations in

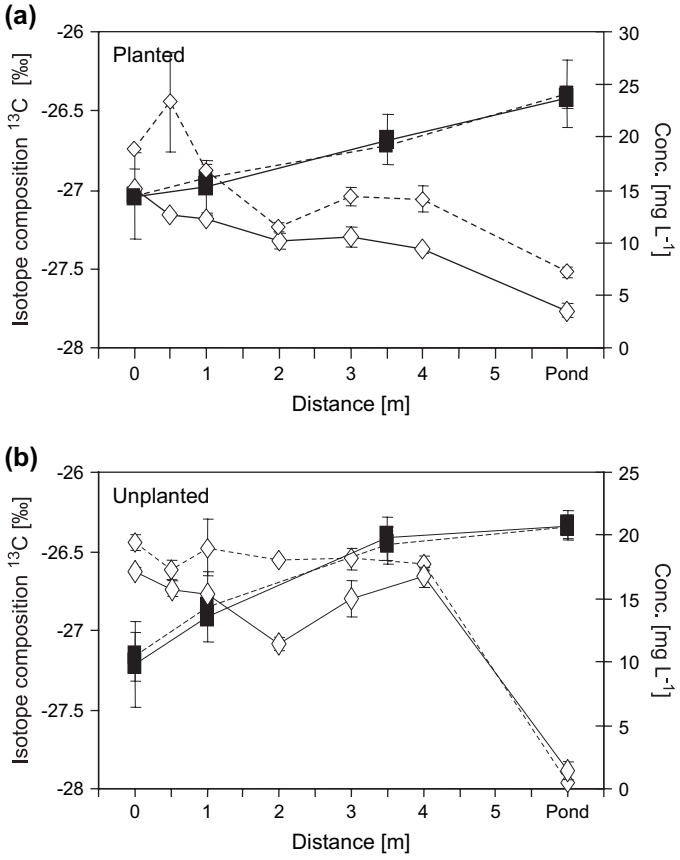

Fig. 2. Concentration and isotopic composition of MCB (diamond, concentration; squares, isotopic composition) in both planted (a) and unplanted (b) segments and for both sampling dates (line, 14.04.2005; dashed, 09.05.2005). Error bars show the standard deviation.

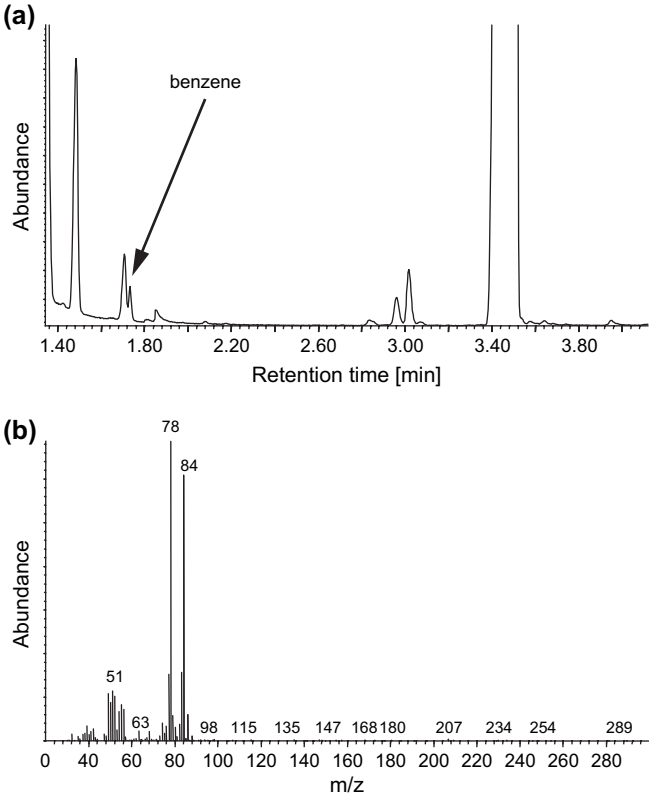

Fig. 3. GC–MS chromatogram (total ion current) of the in situ microcosm extract showing the ^{13}C-labelled benzene metabolite at retention time of 1.73 min and corresponding mass spectrum (a) with the molecule mass of $m/z = 84$ for [$^{13}C_6$] benzene and m/z 78 for [^{12}C] benzene (b).

the microbial community. No systematic differences were observed between the oxic ponds and the anoxic segments of the wetland. However, the lowest quantity of fatty acids was retrieved from the microcosms exposed at 4.5 m in the soil compartments, and the highest accumulated biomass was retrieved from the microcosms deployed in the pond of the unplanted side of the wetland (Fig. 4).

The fatty acid patterns were dominated by high amounts of the saturated hexa- (C16:0) and octadecanoic (18:0) and the monounsaturated hexa- (C16:1) and octadecenoic (C18:1) acids which are common fatty acids in bacteria. The tetra- (C14:0), pentadecanoic (C15:0) and eicosanoic (C20:0) acids, the *iso* and *anteiso* isomers of C15:0 as well as the unsaturated C18:2 were present in lower abundance and could not be detected in all samples (Fig. 4).

The variation of the geochemical parameters down gradient of the inflow was not related to a distinct change in fatty acid composition in both segments. No systematic differences in the fatty acid patterns were found between the planted and unplanted segments. Globally, no clear indications of microbial community changes were obtained on the basis of the fatty acid composition. In this experiment, fatty acid patterns were probably not sensitive enough to reflect changes in the microbial communities as a function of variation in geochemical conditions within the constructed wetland.

3.3.2. Isotope signatures of fatty acids

The total lipid fatty acids extracted from the in situ microcosms displayed some differences in the incorporation of ^{13}C into fatty acids. This was particularly obvious when comparing the samples from the soil compartments and the ponds. The isotope composition of fatty acids ($\delta^{13}C_{FA}$) extracted from the BACTRAPs amended with [$^{13}C_6$]-MCB ranged between −39‰ and 7244‰ (Table 2). An enriched ^{13}C signature of fatty acids (>0‰) can only stem from the microbial utilisation of [$^{13}C_6$]-MCB as a carbon source. In contrast, fatty acids with an isotope signature lower than −20‰ showed the typical natural abundance of $\delta^{13}C_{FA}$ found in soil and aquifer material (Pelz et al., 2001; Pombo et al., 2002). Fatty acids derived from parallel microcosms with non-labelled MCB or from non-amended in situ microcosms displayed an isotope signature ranging from −24‰ to −54‰ (data not shown). This represents a typical isotope composition of lipids derived from microorganisms feeding on organic substrates with natural isotope composition.

Generally, all fatty acids extracted from the in situ microcosms exposed in the ponds showed enrichment in ^{13}C (65‰ to 7244‰), which was generally of higher intensity than in the soil samples (−39‰ to 1832‰). In contrast,

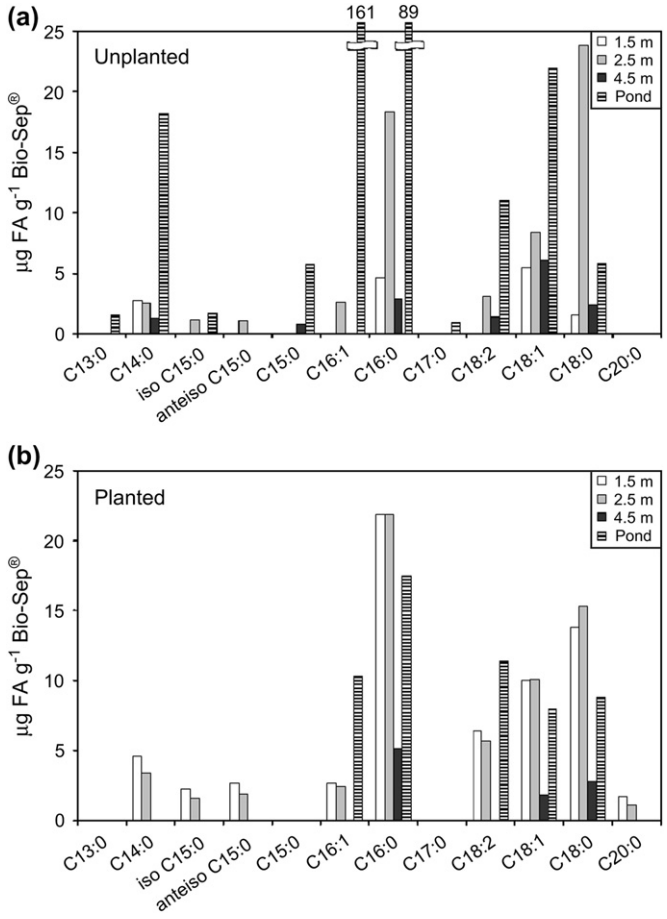

Fig. 4. Absolute abundance of extracted fatty acids [µg FA per gram Bio-Sep® beads] from in situ microcosms exposed in the soil compartment at different distance from the inflow and ponds at the unplanted (a) and planted (b) segments of the constructed wetland.

only fatty acids with up to 16 carbon atoms showed ^{13}C incorporation in the soil samples with highest enrichment in C16 species. Fatty acids with longer carbon chains such as C18:0 or C18:1 displayed no enrichment in ^{13}C (−22‰ to −39‰), indicating that the microbial community was not exclusively growing on the [$^{13}C_6$]-MCB. Comparing the samples from the planted and unplanted segments no significant differences in $\delta^{13}C_{FA}$ were observed. Therefore, the putative impact of plants on the microbial community involved in MCB degradation could not be assessed using our test system.

Some fatty acids can be used as biomarker to identify specific groups of microorganisms (Kaur et al., 2005; Zelles, 1999). In some of the samples labelled *iso* and *anteiso* branched fatty acids with 15 carbon atoms could be identified, indicating that Gram-positive bacteria were involved in the biodegradation of MCB. Labelled C18:2 was only found in samples of the ponds. Linoleic acids (C18:2) can serve as a biomarker for fungi or other eukaryotic organisms (Lösel, 1988) and their presence may lead to the hypothesis that grazing organisms, not involved in the biodegradation of MCB, may feed on the microbial biofilm. However, the applied GC–MS procedure did not allow conclusive identification of the position of the double bonds.

4. Discussion

The geochemical parameters indicated the overall prevalence of anoxic conditions associated with iron mobilisation in the soil parts of the wetland, whereas an aerobic milieu characterised the ponds. In the in situ microcosm experiments, the level of incorporation of labelled carbon into bacterial biomass was used as direct indicator of in situ MCB degradation. Interestingly, the analysis of the BACTRAPs incubated in the ponds revealed fatty acids patterns and ^{13}C incorporation levels differing from the ones retrieved from the soil compartments. The higher ^{13}C incorporation level observed in the ponds of both segments is indicative of a more effective microbial transformation of the [$^{13}C_6$]-MCB under the prevailing aerobic conditions. Along with a significant accumulation of biomass on the microcosms retrieved from the ponds, these results suggest that some change in the microbial community dynamics may operate between the anoxic soil compartments and the more aerobic ponds. Moreover, the fact that all the extracted fatty acids showed incorporation in ^{13}C suggests

Table 2
Carbon isotope composition of fatty acids extracted from in situ microcosms incubated for 6 weeks with $^{13}C_6$ labelled monochlorobenzene at 1.5, 2.5 and 4.5 m from the inflow point as well as at the pond

[m] From inflow fatty acid	Planted $\delta^{13}C_{FA}$ [‰]				Unplanted $\delta^{13}C_{FA}$ [‰]			
	1.5	2.5	4.5	Pond	1.5	2.5	4.5	Pond
C13:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	118
C14:0	−6	40	n.d.	n.d.	138	204	116	238
iso C15:0	121	89	n.d.	n.d.	n.d.	742	n.d.	1597
anteiso C15:0	23	68	n.d.	n.d.	n.d.	3	n.d.	n.d.
C15:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−37	252
C16:1	411	453	n.d.	7244	n.d.	1832	n.d.	806
C16:0	2	9	93	1711	260	95	192	639
C17:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	242
C18:2	−31	−30	n.d.	65	n.d.	−27	−30	216
C18:1	−30	−29	−28	276	−23	−23	−24	175
C18:0	−27	−28	−24	449	−22	−27	−26	266
C20:0	−37	−39	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected.

that the microbial community established on the microcosms was mostly involved in contaminant degradation. However, it should be considered that cross-feeding by metabolites or recycling of dead biomass within the biofilm may also channel labelled carbon into individual members of the microbial community, explaining the variation in the labelling of specific fatty acids. In general, fatty acids displaying a higher incorporation of ^{13}C were very likely synthesised by organisms feeding on [$^{13}C_6$]-MCB, whereas organisms synthesising non-labelled fatty acids were likely not involved in the degradation of the labelled MCB and used different carbon sources. The analysis of the composition of total fatty acid fractions showed that this method might not be sensitive enough for investigating detail changes in microbial communities between soil and water compartments as well as down gradient the flow path.

Additionally, the MCB degradation processes in the wetland were investigated by carbon stable isotope composition analysis. A correlation between decreasing MCB concentration and a shift in the carbon isotope signature towards the heavier isotope was visible along the flow path, suggesting the degradation of MCB. Toxic effects of MCB on the MCB-degrading population can be reasonably excluded at the observed range of concentration values (Fritz et al., 1992; Vogt et al., 2002, 2004).

In the transition from the soil compartments to the ponds, a substantial contaminant mass depletion without concomitant isotope enrichment was observed. In this open system, it is likely that part of the contaminant may partition into the atmosphere, affecting the MCB concentration values without generating a significant isotope shift. Moreover, under oxic-prevailing conditions, several bacteria have the ability to use MCB as sole carbon and energy source, and may putatively adopt the well-known and described aerobic degradation pathways (Van Agteren et al., 1998). These bacteria may degrade MCB at faster rate than the degrading bacteria associated with anoxic conditions, contributing to the observed contaminant mass decrease. These oxygen-driven degradation reactions would lead to MCB mass decrease without a significant associated isotope effect (Kaschl et al., 2005). In parallel, biogeochemical processes such as oxidation of ferrous iron or mineral surfaces may compete for oxygen (Ehrlich, 1998; Søgaard et al., 2001; Warren and Haack, 2001), leading to transient conditions and oxygen gradients, which may affect the composition of the existing microbial community and rate of degradation reactions. However, the anaerobic degradation pathway of MCB is not elucidated yet.

In the anoxic soil compartments, two major hypothetical degradation pathways would come into consideration: (1) reductive dechlorination of MCB; and (2) degradation of MCB as an electron donor molecule. First, an initial dechlorination of MCB, followed by the degradation of benzene, is likely to occur in the soil compartments. Indeed, the presence of ^{13}C-labelled benzene detected in all the microcosms along with the detection of low benzene concentrations suggested that MCB is degraded reductively to benzene under anoxic conditions prior mineralisation. This pathway would suggest a similar pattern as observed previously by Nowak et al. (1996). The pH values were, however, constant, and the relatively high background level of chloride in the groundwater hindered the direct verification of MCB degradation by an increasing chloride concentration along the flow path. Bacterial breakdown of benzene could lead to the formation of benzoate or phenol as intermediates (Chakraborty and Coates, 2005; Edwards and Grbić-Galić, 1992; Lovley, 2000; Phelps et al., 2001; Ulrich et al., 2005), which were not detected in this study. Soluble organic carbon species, which could serve as electron donors for dechlorination processes, were found in low concentration in the unplanted segment, which might effectively limit the extent of degradation. Conversely, dechlorination activity in the planted segment may be partly related to the abundance of hydrogen and reduced organic acids such as acetate and propionate (Holliger et al., 1992; Middeldorp et al., 1997).

Reductive dechlorination reaction of MCB to benzene is expected to be associated with a pronounced primary isotope effect (Griebler et al., 2004). In contrast, the isotope composition presented in this study displayed a slight but significant enrichment ranging from 0.4 to 0.7 δ units, in the soil compartments of the planted and the unplanted segments, respectively. However, the isotope effect at zones of preferential in situ degradation reaction can be substantially higher. If other non-fractionating processes such as sorption and volatilisation also contribute to a decrease in MCB concentrations, the isotope effect upon in situ degradation is expected to be relatively high. The observed isotope effect points to dominating anaerobic processes, as inferred by the estimation of biodegradation levels over the flow path. In the planted segment, the oxygen supplied by the plant at the rizosphere level may favour the establishment of aerobic zones. Although this process is not relevant for the electron budget, oxygen may contribute to the MCB degradation reactions, leading to MCB decrease without concomitant isotope effect. A fractionating anaerobic process and a less fractionating aerobic process may both contribute to in situ degradation, resulting in a mixed overall fractionation at the planted segment. Conversely, the isotopic composition shift observed at the unplanted segment suggests the occurrence of a more fractionating process.

Alternatively, MCB may be degraded as an electron donor molecule under ferric iron- or sulphate-reducing conditions. Anaerobic oxidation of benzene under these conditions has been previously observed (Anderson et al., 1998; Anderson and Lovley, 2000). For instance, geochemical footprints of iron reduction processes were found, and the ferrous iron mobilisation was increasing as a function of the flow path. Dissolved Fe in pore waters can be a result of different processes such as Fe(III) reduction (Lovley, 1991, 1997), pyrite oxidation (Lord and Church, 1983), or Fe complexation (Luther et al., 1996). Fe(II) may be precipitated with sulphide originating from sulphate reduction activity or form complexes. Therefore the concentrations may not reflect the true extent of iron reduction in the presence of sulphate reduction. A low extent of sulphate reduction process and the availability of reactive iron may prevent accumulation of H_2S in the near-neutral conditions of the wetland. A MCB mineralisation by sulphate reduction contributing to the contaminant mass

decrease is feasible and may be possible. However, due to the high concentrations of sulphate in the supplied water, a reliable estimation of the extent of sulphate reduction could not be carried out. However, a reduction of ferric iron directly linked stoichiometrically to MCB oxidation in the wetland may theoretically account for a MCB mass decrease of about 18% between the inflow and the outflow of the system at both the planted and unplanted segment.

Additionally, other unknown degradation pathways cannot be excluded. These hypotheses will require further detailed investigations, along with further isolation and identification of microorganisms involved in the anaerobic MCB degradation in wetlands treating MCB contaminated water.

5. Conclusion

The integrated approach provided evidence for in situ MCB biodegradation in both, soil compartments and ponds of the planted and unplanted segments of a horizontal subsurface flow constructed wetland. This was supported by isotopic fractionation analysis, combined with in situ microcosm experiments, which can be utilized to document further the in situ degradation of MCB and other contaminants in wetland systems. Further investigations to elucidate the microbial degradation of MCB, facilitated by an integrated approach and combined with a high resolution sampling, are required to evaluate zones of enhanced in situ biodegradation of MCB and to optimise wetland systems treating contaminated groundwater.

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Anhang H

Biodegradation of chlorobenzene in a constructed wetland treating contaminated groundwater (2007)

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Biodegradation of chlorobenzene in a constructed wetland treating contaminated groundwater

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Abstract Monochlorobenzene (MCB) is an important groundwater contaminant world-wide. In this study, a horizontal subsurface flow constructed wetland with an integrated water compartment was fed with MCB contaminated groundwater originating from the local aquifer. Analysis of spatial concentration dynamics of MCB and oxygen was combined with isotope composition analysis of MCB for assessing *in situ* biodegradation. Removal of MCB was most effective in the upper layer of the soil filter, reaching up to 77.1%. Trace oxygen concentrations below 0.16 mg L^{-1} were observed throughout the wetland transect, suggesting a considerable limitation of aerobic microbial MCB degradation. Enrichment of ^{13}C in the residual MCB fraction at increasing distance from the inflow point indicated microbial MCB degradation in the wetland. The observed isotope shift was higher than expected for aerobic MCB degradation and thus pointed out a significant contribution of an anaerobic degradation pathway to the overall biodegradation.
Keywords Biodegradation; carbon isotope fractionation; constructed wetland; monochlorobenzene; oxygen

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Introduction

Monochlorobenzene (MCB) was released into the soil at many chemical production sites as a result of its sustained production and use over several decades. It is encountered worldwide as a groundwater pollutant and persists in the essentially anaerobic aquifer at the large-scale contaminated site in Bitterfeld, Germany (Heidrich *et al.*, 2004).

The major mechanism of aerobic MCB degradation is dechlorination, usually initiated by dioxygenative hydroxylation (Vogt *et al.*, 2004). Only some evidences of MCB transformation under anoxic conditions have been presented yet (e.g. Nowak *et al.*, 1996). Recently, indications for anaerobic MCB degradation processes were found in the Bitterfeld aquifer on the basis of isotope fractionation patterns (Kaschl *et al.*, 2005). Kinetic isotope fractionation processes may be employed to demonstrate the biological transformation of organic contaminants (Richnow *et al.*, 2003a). A substantial enrichment of ^{13}C in the non-degraded fraction in the course of a contaminant plume indicates microbial degradation, as dilution and sorption do not significantly affect the isotope composition of contaminants (Harrington *et al.*, 1996; Slater *et al.*, 2000).

Wetland systems represent an effective and inexpensive way to treat groundwater polluted with organic compounds by taking advantage of the geochemical and biological processes (Haberl *et al.*, 2003). Indeed, effective degradation of chlorinated organics has been observed in wetland rhizospheres (Anderson and Walton, 1995; Lorah and Olsen, 1999). Anaerobic and aerobic zones exist in close proximity in the rhizosphere of wetland plants as a result of oxygen release through the roots (Stottmeister *et al.*, 2003).

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This potentially leads to a considerable enhancement of the degradation potential for different organic contaminants.

Little is known about the behaviour of chlorinated benzenes and the relevant removal processes in constructed treatment wetlands. In general, most investigations on phyto-remediation of chlorinated hydrocarbon contaminated waters and soils were carried out under laboratory conditions using micro- or meso-scale systems. Field investigations in pilot or full-scale treatment wetlands are missing. A greater understanding of contaminant removal processes is required to reliably predict the retention and transformation of MCB in wetland systems. Especially, knowledge about oxygen availability and distribution is essential to evaluate the contribution of aerobic microbial degradation to contaminant removal. Information on vertical and longitudinal variations of contaminant and oxygen concentrations in constructed wetlands is presently limited. Biodegradation, as the only process leading to sustainable contaminant reduction in wetland systems, needs to be assessed *in situ*. In this study, we investigated the spatial variations of oxygen and MCB concentrations in combination with an evaluation of *in situ* biodegradation in a wetland treating MCB contaminated groundwater.

Methods

Experimental setup

The pilot-scale constructed wetland was built at the SAFIRA (remediation research in regionally contaminated aquifers) groundwater research site in Bitterfeld, Saxony-Anhalt (Germany). At the site, the groundwater is mainly contaminated by MCB with maximum concentrations of 22 mg L^{-1} (Vogt *et al.*, 2002). The constructed wetland was designed as a horizontal subsurface flow system and consisted of a stainless steel container measuring 6 × 1 × 0.7 m. It was filled to a height of 0.5 m and a length of 5 m with the local aquifer material, leaving the last metre as an open water compartment. The aquifer material used as filter material for the wetland consisted of sand with 30% gravel and 4% silt. Due to residues of the local lignite seam, TOC varied between 1.5 and 2.0%. The iron content of the sediment ranged from 0.8 to 1.3%. The soil filter was planted with common reed (*Phragmites australis (Cav.)*) and was continuously supplied with anaerobic contaminated groundwater at a flow rate of 4.7 L h^{-1}. The water level was adjusted to 0.4 m by a float vale in the water compartment; water volumes pumped off were controlled by a flow meter.

Sampling

The current long term investigation was carried out from November 2004 to October 2005. Samples were taken at 0.5, 1, 2, 3 and 4 m distance from the inflow at 0.3, 0.4 and 0.5 m depth of the soil filter. The water compartment was sampled in 0.3 m depth. Water sampling was carried out with stainless steel lances (3.5 mm inner diameter) and peristaltic pumps at a sampling rate of 78 mL min^{-1}. For isotope composition analysis, pore water samples were collected in April and May 2005 at 0, 1 and 3.5 m from inflow and in the water compartment at 0.5 m depth. NaOH pellets were added to inhibit microbial growth. All water samples were stored without headspace at 4 °C until analysis.

Analysis

MCB concentrations were analysed in duplicate by headspace GC-FID (HP 6890 with HP 7694 autosampler, Agilent technologies, Palo Alto, USA) using a HP-1 column (30 m × 32 mm × 5 μm). The following temperature program was applied: 45 °C (1 min), 20 °C min^{-1} to 200 °C (2.5 min), 65 °C min^{-1} to 250 °C (1 min). The detector temperature was 280 °C. Helium served as carrier gas with a constant flow of 1.7 mL min^{-1}. Samples

were shaken for 1 h at a temperature of 60 °C prior to injection of 1 mL headspace with split 1:5.

Oxygen concentrations were measured in flow through mode using an optical oxygen trace sensor system (sensor FTC-TOS7 and instrument FIBOX-3-trace, PreSens, Regensburg, Germany).

Samples for carbon isotope composition analysis of MCB were extracted within 24 h using 2 mL n-pentane as described previously (Richnow *et al.*, 2003b). The carbon isotope composition was measured with a gas chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) consisting of a GC unit (HP 6890, Agilent technologies, Palo Alto, USA), a combustion device (Finnigan MAT GC III) with water-removal assembly (Nafion® membrane, 50 cm long, T = 0 °C) and a mass spectrometer (Finnigan MAT 252) (ThermoFinnigan, Bremen, Germany). Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. One μL of the n-pentane extract was injected at 250 °C with split 1:10 and separated on a capillary column (Zebron ZB-1, 60 m × 0.32 m × 1 μm; Phenomenex, Torrance, USA). The following chromatographic conditions were applied: injector temperature 250 °C, oven temperature program: 40 °C (1 min), 4 °C min^{-1} to 150 °C, 20 °C min^{-1} to 250 °C (2 min). Samples were measured in triplicate. The carbon isotope composition is reported in the delta notation as $\delta^{13}C$ values [‰] relative to Vienna Pee Dee Belemnite standard (V-PDB, IAEA Vienna).

Results and discussion

Spatial concentration dynamics of MCB were determined as a function of the distance from inflow and depth of the soil filter (Figure 1). A strong relationship between average concentration decrease and the investigated depth of the soil filter was observed. In the upper layer (0.3 m depth), the mean removal efficiency was 77.1% after 4 m of the flow path. Concentration decrease was much lower in deeper zones of the soil filter, reaching an average of 52.3 and 37.1% at 0.4 and 0.5 m depth, respectively. The more effective removal in the upper soil layer may be attributed either to emission of the contaminant from the soil surface or to increasing aerobic microbial MCB oxidation supported by elevated oxygen concentrations in this zone. Higher oxygen concentrations are possibly a result of diffusion of atmospheric oxygen or of oxygen input from plant roots into the upper soil zone. However, most of the residual contaminant charge was eliminated in the adjacent water compartment, probably due to volatilisation and aerobic microbial degradation. Keefe *et al.* (2004) investigated the removal efficiency of a large-scale

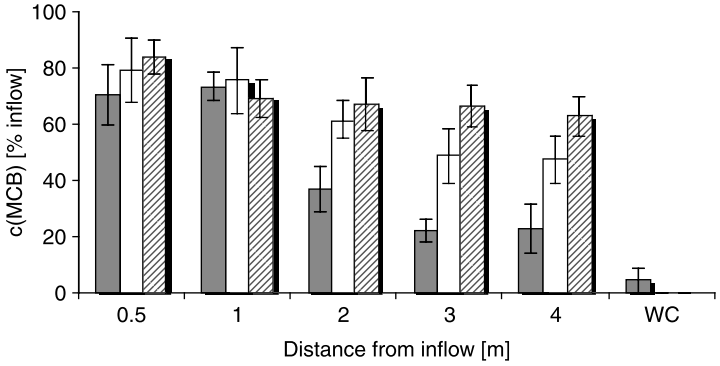

Figure 1 Relative MCB concentrations in percent of inflow concentration as a function of the distance from inflow at 0.3 (grey), 0.4 (white) and 0.5 m (grey-white) depth of the soil filter and in the water compartment (WC) (average of 14 measurements, error bars represent standard deviations)

constructed wetland regarding 1,4-dichlorobenzene with an initial concentration of 0.74 μg L^{-1}. The removal efficiency was found to range from 63 to 87%. Although the initial concentrations were more than four orders of magnitude higher in our experiment, the relative removal rates in the upper layer of the wetland were similar to the ones reported by Keefe *et al.* (2004).

Spatial concentration dynamics of oxygen were determined in the soil filter of the constructed wetland to investigate the prevailing redox conditions, and thus the possibility of aerobic microbial MCB degradation. In general, very low oxygen concentrations were observed throughout the wetland profile (Figure 2). Taking into account the oxygen demand for mineralisation of MCB (7 mol O_2 per mol MCB) and the high inflow concentrations of the contaminant (22 mg L^{-1}), a strong oxygen limitation can be concluded. Oxygen released from plant roots may be rapidly consumed by microbial processes and thus will not lead to entirely aerobic conditions. Nevertheless, in the direct vicinity of the roots, aerobic compartments may exist and contribute to aerobic MCB degradation. Only on the first part of the flow path (up to 1 m distance from inflow), relatively higher average concentrations were observed, reaching a maximum of 0.16 mg L^{-1} dissolved O_2 in the upper layer of the soil filter. This may be a result of oxygen input by plants as the plant density was also highest in this zone of the wetland. The spatial heterogeneity of the plant stock can be attributed to limitation of nutrients, in particular of ammonia and phosphorus (data not shown).

To assess *in situ* biodegradation and to gain evidence for possible microbial degradation pathways, the carbon isotope fractionation of MCB in the wetland was investigated. A decrease in MCB concentrations over the transect as shown in Figure 1 was systematically associated with a significant enrichment in ^{13}C (Table 1). This indicates that MCB was biodegraded *in situ*, assuming it has an isotopically homogeneous source and sorption and dilution do not significantly affect the isotopic composition.

MCB showed a maximum isotope shift of 0.65‰. Kaschl *et al.* (2005) found that the aerobic degradation of MCB only results in a very low fractionation effect, whereas the reductive dechlorination of e.g. trichlorobenzene exhibits a higher isotope fractionation (Griebler *et al.*, 2004). The fact that a considerable isotope shift associated with concentration decrease of MCB was observed in the actual experiments suggests the predominance of an anaerobic degradation pathway in the wetland. An isotope shift of more

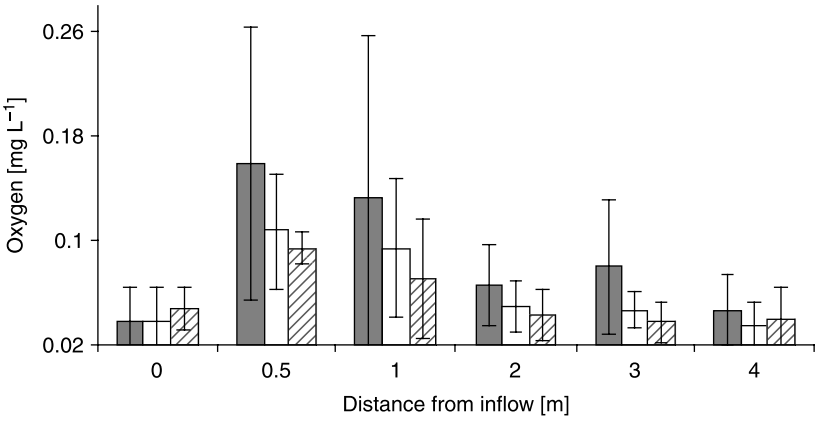

Figure 2 Oxygen concentrations as a function of the distance from inflow at 0.3 (grey), 0.4 (white) and 0.5 m (grey-white) depth of the soil filter (average of seven measurements taken over the study period, error bars represent standard deviations)

Table 1 Isotopic composition of MCB in different distances from the inflow and in the water compartment (WC) and overall isotope shift $\Delta^{13}C$ for two sampling dates

Date	13.04.05			09.05.05		
Distance [m]	$\delta^{13}C$ [‰]	Standard deviation	$\Delta^{13}C$ [‰]	$\delta^{13}C$ [‰]	Standard deviation	$\Delta^{13}C$ [‰]
0	−27.04	0.27		−27.04	0.18	
1	−26.98	0.16		−26.92	0.04	
3.5	−26.67	0.16		−26.71	0.03	
WC	−26.42	0.07	0.62	−26.39	0.22	0.65

than 4% was observed between the fringes and the centre of a MCB plume in the anaerobic aquifer in Bitterfeld (Kaschl *et al.*, 2005). In the heterogeneous wetland system, processes such as sorption, dilution, volatilisation and plant uptake may contribute to the decrease in contaminant mass without affecting the isotope composition. This would lead to an underestimation of isotope fractionation. Due to the lack of a laboratory-derived fractionation factor for anaerobic MCB degradation, the respective contribution of anaerobic degradation processes to overall *in situ* biodegradation in the wetland could not be quantified. Nevertheless, the qualitative evidence for anaerobic MCB biodegradation in the wetland is a step towards greater understanding of MCB elimination in wetland systems. The mechanistic identification and the quantification of aerobic and anaerobic MCB degradation processes should be addressed in further experiments.

Conclusions

The actual data show that MCB concentration decreased along the wetland transect, suggesting the occurrence of active removal processes in the system. The removal was most effective in the upper layer of the soil filter. Low integral oxygen concentrations prevailed throughout the wetland and probably limited aerobic microbial degradation processes. It is likely that aerobic processes were restricted to the vicinity of the plant roots. Enrichment of ^{13}C in the residual contaminant fraction provided evidence for *in situ* MCB biodegradation and suggested that anaerobic microbial degradation processes played a relevant role. This is of great interest as knowledge about the mechanism and efficiency of anaerobic MCB degradation is still very limited in general. Furthermore, these findings confirm the multifunctional character of constructed wetlands. The interactions of plants, soil and microorganisms create an extremely heterogeneous environment and biogeochemical gradients in the wetland system. The input of oxygen into the rhizosphere performed by wetland plants leads to the formation of aerobic zones near the root surface so that aerobic microorganisms should prosper in the root zone. As the oxygen is rapidly consumed for oxidation of organic carbon, anoxic conditions prevail in the bulk soil and anaerobic microorganisms can establish there. This allows the simultaneous occurrence of aerobic as well as anaerobic MCB biodegradation pathways in the constructed wetland.

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Anhang I

Applicability of Stable Isotope Fractionation Analysis for the Characterization of Benzene Biodegradation in a BTEX-contaminated Aquifer (2007) und Supporting Information

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Applicability of Stable Isotope Fractionation Analysis for the Characterization of Benzene Biodegradation in a BTEX-contaminated Aquifer

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In recent years the analysis of stable isotope fractionation has increasingly been used for characterizing and quantifying biodegradation of contaminants in aquifers. The correlation of carbon and hydrogen isotope signatures of benzene in a BTEX-contaminated aquifer located in the area of a former hydrogenation plant gave indications that biodegradation mainly occurred under anoxic conditions. This finding was consistent with the investigation of hydrogeochemical conditions within the aquifer. Furthermore, the biodegradation of benzene was calculated by changes in carbon isotope signatures using the Rayleigh-equation-streamline approach. Since contaminant concentrations can be also affected by nonisotope-fractionating abiotic processes such as dilution, volatilization, or irreversible sorption to the aquifer matrix, the Rayleigh-equation-streamline approach was adjusted for scenarios assuming that biodegradation and abiotic processes occur either consecutively or simultaneously along a groundwater flow path between contaminant source and sampling well. The results of the scenarios differed significantly, indicating that an abiotic process (typically dilution) causes a decrease in benzene concentration within the investigated aquifer transect. This comparison of results derived from the different scenarios can help to identify whether biodegradation is the predominant process for decrease in contaminant concentration. However, for a proper quantification of biodegradation, the temporal sequence between biodegradation and dilution needs to be known. The uncertainty associated with the quantification of pollutant biodegradation by the Rayleigh-equation-streamline approach increases when nonisotope-fractionating abiotic processes cause a significant decrease in contaminant concentrations.

Introduction

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are groundwater pollutants of particular environmental concern, since these compounds represent a significant health risk due to their high abundance and toxicity (*1*, *2*). It has been shown that all BTEX compounds can be biodegraded under oxic and anoxic conditions (see refs *3* and *4* for reviews). Biodegradation is the most significant process leading to a decrease in BTEX concentrations in groundwater coupled with a sustainable mass reduction. Therefore, the evaluation of in-situ BTEX biodegradation is essential for the implementation of groundwater management strategies such as natural attenuation (NA) (*5*). In recent years, stable isotope fractionation analysis has gained more and more attention as a tool for characterizing and assessing in-situ biodegradation of organic pollutants in contaminated aquifers (see ref *6* for a review). This concept relies on the fractionation of stable isotopes during the microbial degradation, leading to an enrichment of heavier isotopes in the residual fraction of a pollutant. Thus, the isotope fractionation of carbon, hydrogen, or other elements involved in the cleavage or formation of chemical bonds during the initial step of microbial transformation can be used as an indicator for the in-situ biodegradation. In many studies, the percentage of contaminant biodegradation was determined by the Rayleigh-equation-based approach (*7*−*14*). Abe and Hunkeler showed that the Rayleigh-equation-based approach for the quantification of contaminant biodegradation is quite robust and always conservative in respect to longitudinal dispersion effects, degrees of biodegradation, plume geometries, and travel times (*15*). Besides for quantification, the simultaneous changes in carbon and hydrogen isotope signatures were used for the characterization of aerobic or anaerobic degradation of methyl *t*-butyl ether (MTBE) in contaminated aquifers (*10*, *13*).

However, effects of abiotic processes on the isotopic signature of the target compound have to be known to characterize biodegradation of organic pollutants in aquifers. No significant changes in the isotopic signature of contaminants occur during the dilution with pristine water (*16*). The isotope fractionation due to volatilization is largely negligible in contaminated aquifers (*17*, *18*). Several laboratory studies showed that sorption of BTEX causes no significant isotope fractionation, leading to the assumption that this process has no influence on the isotope signature of a pollutant dissolved in groundwater (*17*, *19*, *20*). However, Kopinke et al. showed that for an aquifer where no biodegradation occurs, sorption can lead to a significant isotope fractionation at the front of an expanding contaminant plume or a breakthrough curve occurring after a short contamination event (*21*). In the case of a stationary contaminant plume with biodegradation activity, the degree of isotope fractionation due to sorption will be smaller and possibly insignificant (*21*). Another process that can affect the isotope signature of pollutants in aquifers is the mixing of groundwater from aquifer zones which exhibit a different degree of biodegradation. This mixing effect leads to a decrease in the observable isotope fractionation of a pollutant (*7*, *8*, *21*). A model to estimate the influence on isotope fractionation due to mixing of groundwater and consequences for the assessment of in-situ biodegradation is given by Kopinke et al. (*21*).

In this study, stable isotope fractionation analysis was used to characterize and assess the in-situ biodegradation of benzene in a highly contaminated aquifer. Based on multilevel sampling, the vertical and horizontal distributions of carbon and hydrogen isotope ratios for benzene were detected within the contaminant plume. By means of the isotope ratios, we intend to determine whether the biodegradation of benzene occurred under oxic or anoxic conditions.

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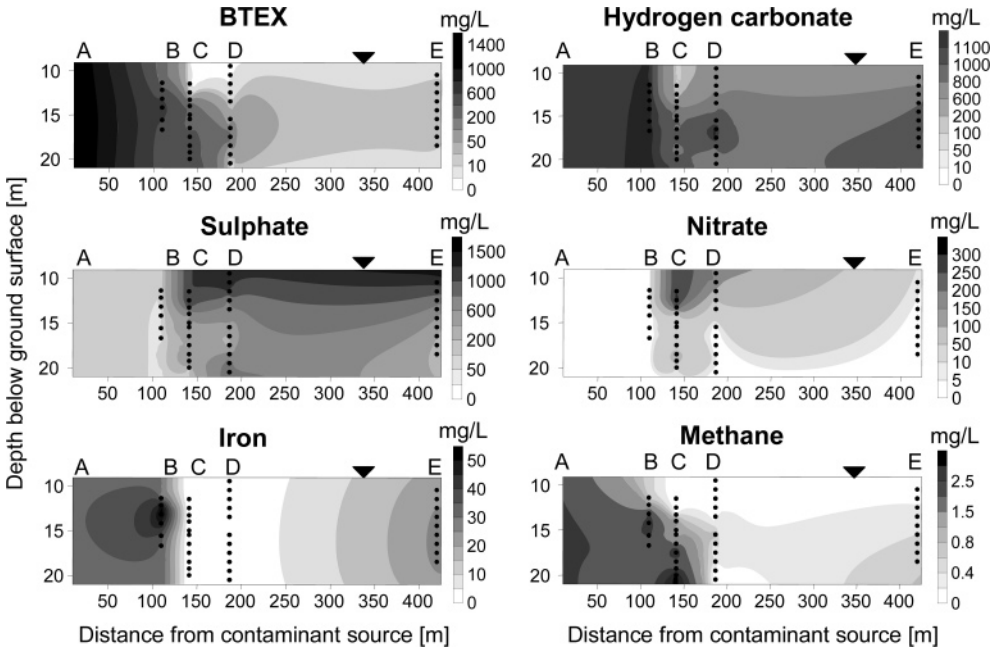

FIGURE 1. Distribution of concentrations of pollutants, electron acceptors, and mineralization products within the investigated aquifer transect. The letters stand for the sampling wells and the black dots for the depths sampled for the analyses of the hydrogeochemical parameters. The black triangle indicates the depth of the groundwater table.

Furthermore, the benzene biodegradation was assessed based on the Rayleigh equation. Since the Rayleigh equation links changes in isotope ratios to concentration shifts, abiotic concentration decreasing processes should be taken into account for the quantification of pollutant biodegradation using the Rayleigh-equation-based approach. Until now, there are a large number of publications also including guidelines from environmental agencies or organizations showing the application of the Rayleigh-equation-based approach for quantification of pollutant biodegradation in contaminated aquifers (e.g., refs *7*−*14*, *22*). Only a few noted that nonisotope-fractionating abiotic processes like dilution can have an effect for the reliability of the assessment of biodegradation based on the Rayleigh equation model. To examine the applicability of the stable isotope fractionation analysis for assessing pollutant biodegradation in contaminated aquifers, we considered various scenarios where, on the one hand, the shift in isotope ratios is only a result of microbial transformation and, on the other hand, the changes in concentrations are influenced not only by biodegradation but also by a nonisotope-fractionating abiotic process.

Materials and Methods

Field Site. The investigated field site is located in the area of a former hydrogenation plant close to the city of Zeitz (Saxony-Anhalt, Germany). The history and hydrogeological conditions of the field site are described in detail in recent studies (*8*, *12*) and in the Supporting Information.

In the near-source areas, the BTEX concentration can exceed 1000 mg/L. The dominant contaminants are benzene and toluene with concentrations up to 950 mg/L and 50 mg/L, respectively. At the test site, the predominant electron acceptor is sulfate (*8*, *12*). In addition, methanogenesis accounts for minor microbial BTEX transformation in the contaminant plume (*8*). Other electron acceptors such as oxygen, nitrate, and ferric iron play only a marginal role for biodegradation processes at the site (*8*, *12*, *23*).

For hydrochemical and isotopic analyses, groundwater samples were taken at several multi-level sampling wells (A, B, C, D, E) along a transect in the upper aquifer extending from a source area to the fringe of the contaminant plume following the main groundwater flow direction (Figure 1 and Figure 1 in the Supporting Information). The sampling and analytical methods are described in detail in the Supporting Information.

Theoretical Background

The carbon and hydrogen isotope ratios were expressed in the delta notation (δ^{13}C and δ^{2}H) in per mil (‰) units according to eq 1. In eq 1, R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C-$

$$\delta^{13}C_{sample} \text{ or } \delta^{2}H_{sample}\ [‰] = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \times 1000 \quad (1)$$

or $^{2}H/^{1}H$-ratios of the sample and an international standard, respectively. Vienna Pee Dee Belemnite (VPDB) was used as the standard for the analysis of carbon isotope signature and Vienna Standard Mean Ocean Water (VSMOW) was used as the standard for the detection of hydrogen isotope ratios (*24*).

For the description of isotope fractionation of biochemical reactions the Rayleigh equation can be used (*25*):

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\epsilon/1000} \quad (2)$$

where R_t, C_t and R_0, C_0 are the stable isotope ratios and concentrations of a compound at a given point in time and at the beginning of a transformation reaction, respectively. The enrichment factor ϵ [‰] provides the link between the changes in stable isotope ratios (R_t/R_0) and the changes in the concentrations (C_t/C_0).

In several studies, the Rayleigh equation was used to quantify the biodegradation of pollutants in contaminated aquifers. An appropriate enrichment factor and the changes in isotope signatures of the contaminant (R_x/R_0) may be used to calculate the decrease in the pollutant concentration due to biodegradation along a flow path between a contaminant source and a sampling well ($f_x = CB_x/C_0$, CB_x is the expected pollutant concentration at a sampling point resulting only

from biodegradation). This calculation results in the quantification of the percentage of biodegradation (B[%]) by the well-known Rayleigh-equation-streamline approach (*7*−*14*):

$$B[\%] = (1 - f_x) \times 100 = \left[1 - \left(\frac{R_x}{R_0}\right)^{(1000/\epsilon)}\right] \times 100 \quad (3)$$

To estimate the amount of biodegraded pollutant in the presence of a nonisotope-fractionating process leading to a decrease of contaminant concentration, three contrasting scenarios were considered. The derivation of the following equations is given in detail in the Supporting Information.

In the first scenario, it was assumed that along the streamline between the contaminant source and the sampling well, biodegradation occurs first then stops, after which a nonisotope-fractionating process decreases pollutant concentration. Thus, the nonisotope fractionating process does not affect the percentage of biodegradation (B[%]), and therefore, it can be calculated by changes in isotope ratios using an appropriate enrichment factor according to eq 3.

For the second scenario, it was assumed that, along a streamline, a nonisotope-fractionating process occurs first and only after its termination biodegradation takes place. Assuming that at the transition from the nonisotope-fractionating process to biodegradation the isotope ratio is equal to the isotope ratio of the contaminant source, the percentage of biodegradation (B[%]) can be quantified as follows:

$$B[\%] = \left(\frac{F_x}{\left(\frac{R_x}{R_0}\right)^{(1000/\epsilon)}} \times \left(1 - \left(\frac{R_x}{R_0}\right)^{(1000/\epsilon)}\right)\right) \times 100 \quad (4)$$

where F_x is the overall extent of concentration decrease along the streamline ($F_x = C_x/C_0$).

The sequence of biodegradation and nonisotope-fractionating process for scenarios 1 and 2 is an unlike situation in contaminated aquifers because both processes take place in parallel. However, these scenarios represent opposite extremes for the effect of a nonisotope-fractionating process on the quantification of biodegradation based on stable isotope analysis. Therefore, they indicate the range of uncertainty caused by the occurrence of a nonisotope-fractionating process at a given site.

In the third scenario it was assumed that biodegradation and a nonisotope-fractionating process occur simultaneously as expected for contaminated aquifers. Assuming that biodegradation and a nonisotope-fractionating process follow first-order kinetics, rates of both processes for pollutant concentration decrease (r_b and r_n, respectively) along a stream line between contaminant source and sampling well are given by

$$r_b = \mu C \quad (5)$$

and

$$r_n = \beta C \quad (6)$$

with C as concentration of a pollutant and μ and β as rate constants of biodegradation and the nonisotope-fractionating abiotic process, respectively. Based on this assumption, the percentage of biodegradation (B[%]) along a streamline between source and a well is given by

$$B[\%] = \frac{\ln\left(\left(\frac{R_x}{R_0}\right)^{1000/\epsilon}\right)}{\ln(F_x)} \times (1 - F_x) \times 100 \quad (7)$$

Furthermore, expressing the relation between the rate for biodegradation and a nonisotope-fractionating abiotic process by the ratio $k = \beta/\mu$, the influence of the nonisotope-fractionating process for the calculation of B[%] along a streamline between the source and a well can be described by

$$B[\%] = \frac{1}{1 + k} \times \left(1 - \left(\left(\frac{R_x}{R_0}\right)^{(1000/\epsilon)}\right)^{1+k}\right) \times 100 \quad (8)$$

Results and Discussion

Hydrogeochemical Conditions and Isotope Fractionation in the Investigated Aquifer. To describe the aquifer conditions under which biodegradation of BTEX occurred, the distribution of pollutants, electron acceptors, and mineralization products were investigated for various multi-level sampling wells along the main groundwater flow direction (Figure 1). The BTEX concentration in the source area (sampling well A) was 1335 mg/L, which exceeded the highest previously detected source concentration of BTEX (approximately 1000 mg/L (*8*, *12*)) significantly. Benzene was the main contaminant with concentrations up to 1310 mg/L. The water solubility of benzene at the temperature measured in the aquifer (12−13 °C) is approximately 1610 mg/L and thus does not differ highly from source concentration of benzene showing the existence of a pollutant phase. Along a monitoring transect down gradient the main groundwater flow direction, BTEX concentrations decreased by up to 96%. Pollutant concentrations were highest between 12 and 17 m below the ground surface and decreased toward upper and deeper zones. Hydrogen carbonate concentrations of up to 1100 mg/L were significantly higher within the transect compared to the groundwater upstream of the contaminant plume (350−650 mg/L) (*8*). The increase in hydrogen carbonate concentration indicated mineralization processes at this site (*8*). The high methane concentrations (up to 4 mg/L) especially in the source area showed that biodegradation occurred under methanogenic conditions. The highest sulfate concentrations (approximately 1500 mg/L) were detected near the groundwater table. Sulfate depletion to concentrations lower than 200 mg/L in the highly contaminated zones, especially in and near the source area, indicating sulfate reduction as the dominant biodegradation process. Further downstream from the contaminant source, nitrate was detected in higher concentrations (up to 275 mg/L) near the groundwater table and decreased toward deeper zones of the aquifer and along the groundwater flow path suggesting microbial nitrate reduction or denitrification. In several zones, dissolved iron could be determined at relatively high concentrations (up to 54 mg/L). At the near neutral pH typical for this site, dissolved iron should mainly occur in the reduced form (Fe(II)). Thus, the observed increase in iron concentration indicates biodegradation under iron-reducing conditions. Significant dissolved oxygen concentrations were not detectable in the investigated aquifer transect suggesting that biodegradation along the transect takes place under anoxic conditions. Similar results were obtained in previous studies for the upper aquifer of the Zeitz test site (*8*, *12*).

For the characterization of the biodegradation in the aquifer transect (Figure 1 and 2), stable isotope fractionation analyses of benzene were carried out since benzene accounted for up to 98% of the BTEX source concentration. The carbon and hydrogen isotope signatures of benzene in the source area (sampling well A) were −28.8 ± 0.5‰ and −147 ± 1‰, respectively. While benzene concentrations decreased by more than 60%, no significant carbon and hydrogen isotope fractionation could be determined at a distance of 110 m from the source area (sampling well B) (Figure 2). Further downstream from the source area, a

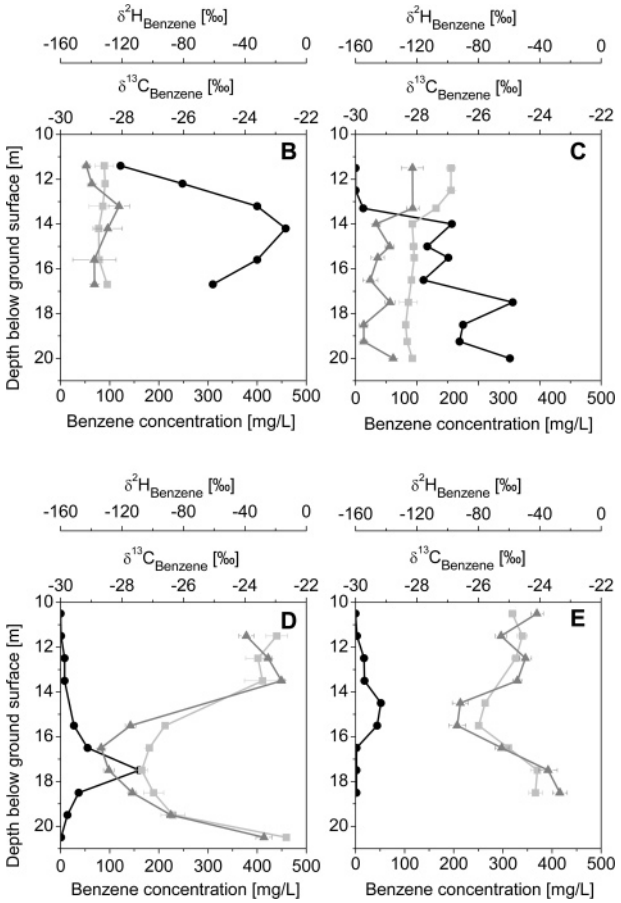

FIGURE 2. Benzene concentrations (black circle) and respective carbon (light gray rectangle) and hydrogen (gray triangle) isotope ratios (given as delta notation) at the various sampling depths of wells B−E. The reproducibilities of $\delta^{13}C$- and $\delta^{2}H$-values are given as error bars and were always smaller than 0.5 and 10‰, respectively. Error bars which are smaller than 0.13‰ for carbon and 2.5‰ for hydrogen are not visible due to the size of the symbols. The relative error of benzene concentration analyses was smaller than ±10‰.

significant ^{13}C- and ^{2}H-enrichment in the residual benzene fraction was detected within the upper part of sampling well C, where benzene concentrations were lower than 20 mg/L. At sampling wells D and E, carbon and hydrogen isotope fractionation was detected at all sampled depths. The ^{13}C- and ^{2}H-enrichment correlated with a significant decrease in benzene concentrations, which can be attributed to biodegradation.

Determination of Benzene Biodegradation Pathways by Isotope Signatures. To investigate under which conditions benzene biodegradation occurred, carbon and hydrogen enrichment factors from the literature (*26*, *27*) were used to determine possible ranges of isotope signature patterns representing aerobic and anaerobic transformation, respectively. The carbon enrichment factors (ϵ_c) for aerobic and anaerobic benzene degradation range between −1.5 and −3.6‰ and show no significant trend for anoxic or oxic conditions. Hydrogen enrichment factors differ significantly for aerobic ($\epsilon_H = -11$ to −13‰) and anaerobic ($\epsilon_H = -29$ to −79‰) benzene degradation. It has been suggested that the initial step of the anaerobic benzene transformation involves a primary hydrogen isotope effect, which means that a C−H bond is broken (*27*). The first attack during aerobic biodegradation of benzene involves no C−H cleavage (*26*). Therefore, only small or no hydrogen isotope effects could be observed for aerobic benzene biodegradation (*26*, *27*).

Carbon and hydrogen isotope ratios (R_x) were calculated by means of the Rayleigh equation (eq 2) using arbitrarily chosen changes in benzene concentrations (f_x), highest and lowest known enrichment factors (ϵ_C, ϵ_H) for aerobic and anaerobic benzene biodegradation, and measured carbon and hydrogen isotope ratios of the benzene source (R_0). The theoretical ranges of isotope signature patterns obtained for aerobic and anaerobic benzene degradation were plotted together with the isotope ratios detected for the sampling wells of the investigated transect (Figure 3). Most of the measured data exhibited carbon and hydrogen isotope signatures which are indicative of anaerobic benzene biodegradation. This is supported by the hydrochemical conditions in the investigated aquifer transect and by recent studies (*8*, *12*, *23*).

Quantification of Benzene Biodegradation. Carbon and hydrogen isotope fractionation can be used for the quantification of pollutant biodegradation. Since sulfate reduction is the predominant electron-accepting process in the upper aquifer of the Zeitz test site (*8*, *12*), enrichment factors derived from benzene biodegradation experiments under sulfate-reducing conditions ($\epsilon_C = -3.6$‰, $\epsilon_H = -79$‰) (*27*) were chosen for the calculation. These are also the factors representing the highest isotope fractionation known from benzene biodegradation experiments, leading to a conservative assessment of biodegradation (*6*, *12*). For the calculation, it was assumed that the enrichment factors did not change along a flow path.

At all sampling depths of well B, carbon and hydrogen isotope ratios were in the range of the variation of the source area; therefore, no calculations were performed for this well. The relative benzene biodegradation B[%] for scenario 1 (eq 3) ranged between <1−42%, 18−84%, and 54−81% for sampling wells C, D, and E, respectively (Table 1). Differences in benzene biodegradation B[%] calculated from carbon and hydrogen isotope signatures were generally smaller than 21% and smaller than 10% for the vast majority of the sampling points. The agreement of calculations derived from carbon and hydrogen isotope signatures shows that analysis of both isotopic ratios provides valuable tools for the assessment of anaerobic benzene biodegradation which was already demonstrated by a field study from Mancini et al. (*14*).

By multiplying the percentage of biodegradation (B[%]) with the benzene concentration in the source area (C_0 = approximately 1310 mg/L), the reduction in benzene concentration due to biodegradation (Δ_B) can be calculated for the streamlines between the contaminant source and each sampling depth of the wells (Table 1). For scenario 1, the highest decrease in benzene concentrations caused by biodegradation exceeds 1000 mg/L. To verify whether such high benzene concentrations could be biodegraded within the transect, an assessment of the biodegradation potential of electron-accepting processes was carried out. For the biodegradation of 1 mg/L benzene, 4.77 mg/L nitrate or 4.6 mg/L sulfate is needed and 21.5 mg/L reduced iron (Fe(II)) or 0.77 mg/L methane is formed, respectively (*28*). Using the highest concentrations of nitrate (275 mg/L), sulfate (1500 mg/L), iron (54 mg/L) and methane (4 mg/L) measured in the transect, a maximum benzene biodegradation potential of up to 400 mg/L can be expected from the electron-accepting processes. Since reduced iron can be precipitated in the presence of sulfide, the potential of electron-accepting processes for benzene biodegradation might be higher than that estimated by the hydrogeochemical indicators. Nevertheless, it is not expected to be much higher because iron reduction does not play an important role for biodegradation at the site (*23*). However, the highest reduction of benzene concentrations due to biodegradation derived from scenario 1 was more than 600 mg/L higher than could be expected from the potential of the electron-accepting processes. Thus,

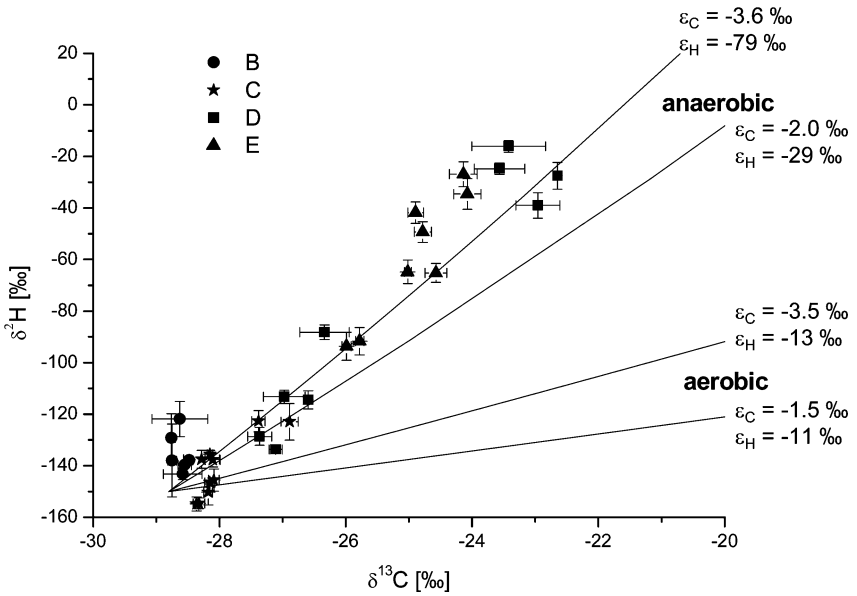

FIGURE 3. Concurrent carbon and hydrogen isotope ratios of benzene measured at the various sampling depths of wells B−E together with isotope patterns for aerobic and anaerobic benzene degradation calculated from published enrichment factors for carbon (ϵ_C) and hydrogen (ϵ_H) as well as the isotope signature of the contaminant source using the Rayleigh equation (eq 2). The reproducibilities of δ^{13}C- and δ^2H-values are given as error bars and were always smaller than 0.5 and 10‰, respectively. Error bars which are smaller than 0.08‰ for carbon and 2.0‰ for hydrogen are not visible due to the size of the symbols.

TABLE 1. Percentage of Benzene Biodegradation (B[%]) and Decrease in Benzene Concentration Due to Biodegradation (Δ_B) for the Various Depths of the Sampling Wells C−E Calculated for Three Scenarios[a]

Well	Depth below ground surface [m]	Scenario 1 (eq 3): source → bio-degradation → nonisotope-fractionating process → sampling point				Scenario 2 (eq 4): source → nonisotope-fractionating process → bio-degradation → sampling point				Scenario 3 (eq 7): source → biodegradation nonisotope-fractionating process → sampling point			
		B [%]		Δ_B [mg/L]		B [%]		Δ_B [mg/L]		B [%]		Δ_B [mg/L]	
		$^{13}C/^{12}C$	$^2H/^1H$	$^{13}C/^{12}C$	$^2H/^1H$	$^{13}C/^{12}C$	$^2H/^1H$	$^{13}C/^{12}C$	$^2H/^1H$	$^{13}C/^{12}C$	$^2H/^1H$	$^{13}C/^{12}C$	$^2H/^1H$
C	11.50	42	30	555	389	<1	<1	<1	<1	6	4	77	50
	12.50	42	n.a.	555	n.a.	<1	n.a.	<1	n.a.	6	n.a.	73	n.a.
	13.30	34	30	441	392	1	1	8	7	9	8	120	104
	14.00	17	<1	227	2	3	<1	41	1	9	<1	112	4
	15.00	18	13	240	167	2	2	33	21	8	6	107	72
	15.50	19	2	246	28	3	<1	44	4	9	1	120	12
	16.50	17	n.d.	218	n.d.	2	n.d.	28	n.d.	7	n.d.	95	n.d.
	17.50	n.d.	13	n.d.	172	n.d.	4	n.d.	48	n.d.	8	n.d.	99
	18.50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	19.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	20.00	17	16	227	204	5	4	66	58	10	9	133	118
D	11.50	81	78	1063	1020	<1	<1	4	4	23	21	305	275
	12.50	78	82	1017	1070	2	3	28	36	29	33	382	433
	13.50	79	84	1029	1095	2	3	29	41	30	35	393	462
	15.50	47	38	615	495	2	1	24	16	16	12	209	157
	16.50	39	18	506	234	3	1	35	12	15	6	193	78
	17.50	34	24	446	310	6	4	83	50	17	11	228	148
	18.50	41	39	537	509	2	2	26	24	14	13	188	176
	19.50	51	57	664	747	1	1	14	19	15	18	202	241
	20.50	83	81	1084	1061	<1	<1	5	4	24	23	321	303
E	10.50	67	77	883	1009	2	3	22	35	23	30	302	396
	11.50	70	69	920	899	2	2	27	25	25	24	332	318
	12.50	68	75	896	978	2	3	27	37	25	29	321	383
	14.50	58	55	760	718	2	1	20	18	19	17	249	229
	15.50	55	54	726	702	1	1	19	18	18	17	236	224
	16.50	66	69	867	902	2	3	32	36	24	26	321	345
	17.50	74	79	971	1037	4	5	50	66	31	36	405	469
	18.50	74	81	965	1063	4	6	52	80	31	39	405	505

[a] For scenario 1, it is assumed that biodegradation occurs before a nonisotope-fractionating process (eq 3); for scenario 2, that a nonisotope-fractionating process takes place before biodegradation (eq 4); and for scenario 3, that both processes proceed simultaneously (eq 7). n.d. is not determined because the isotope ratio was in the range of variation of the contaminant source. n.a. is not assessed due to loss of sample.

scenario 1, representing the conventional Rayleigh-equation-streamline approach (eq 3), probably leads to an overestimation of benzene biodegradation along the flow paths between contaminant source and the various depths of the sampling wells.

For scenarios 2 (eq 4) and 3 (eq 7), the decrease in benzene concentrations due to biodegradation (Δ_B) calculated for the streamlines between the contaminant source and the sampling depths of the wells was lower than 83 and 505 mg/L, respectively (Table 1). Thus, both scenarios provided as-

sessments for benzene biodegradation which are more consistent with the potential of electron-accepting processes (up to 400 mg/L). However, the benzene biodegradation derived from scenario 2 was only approximately one-fifth of the potential of the electron-accepting processes, which indicates an underestimation of benzene biodegradation by scenario 2.

Scenarios 1 and 2 represent opposite extremes for the effect of a nonisotope-fractionating process on the quantification of biodegradation based on stable isotope analysis. Thus, differences between results for these scenarios indicate the range of uncertainty caused by the occurrence of a nonisotope-fractionating process at a given site. As the benzene biodegradation calculated for these two scenarios differed highly (Tab. 1), it can be assumed that another process influenced the decrease of benzene concentrations. At the site, it is unlikely, due to the low carbon content (<0.1%), that irreversible sorption leads to a significant decrease in pollutant concentrations (*29*). Since benzene could only be detected in minor concentrations (<1 mg/L) approximately one meter below the groundwater table along the transect, it can be assumed that only a very small proportion of the benzene might be affected by volatilization. Thus, volatilization can be neglected as a process causing a significant decrease of benzene concentrations. Since irreversible sorption and volatilization obviously only led to a minor reduction in benzene concentration, dilution with pristine water is apparently a significant process causing a decrease in pollutant concentration.

The differences for benzene biodegradation calculated by scenarios 1 and 2 were always higher for depths within the fringe of the benzene plume compared to depths within the centerline (Table 1). This is consistent with the assumption of a higher dilution with pristine water at the fringe of the benzene plume compared to its centerline. To include dilution effects into the quantification of biodegradation the temporal evolution of both processes along a streamline needs to be known. In general, the simultaneous occurrence of processes as assumed for scenario 3 is a much more realistic than the strictly sequential order of processes used for scenarios 1 and 2. This is supported by scenario 3 providing the best estimate of biodegradation compared to the degradation potential of the electron-accepting processes. However, the assumption made for scenario 3 (e.g., biodegradation and the nonisotope fractionating process both following first-order kinetics) are still simplifications, and to obtain a reliable assessment for benzene degradation at the site a more detailed knowledge of the influence of dilution on the decrease of benzene concentration would be necessary.

To illustrate the influence of a nonisotope-fractionating process for the accuracy of quantification of pollutant biodegradation via the conventional Rayleigh-equation-streamline approach (eq 3), scenario 3 was expanded by a parameter k expressing the relation between the rate for biodegradation and a nonisotope-fractionating process (eq 8). With increasing influence of the nonisotope-fractionating process, the conventional Rayleigh-equation-based approach (eq 3) leads to an overestimation of pollutant biodegradation along a streamline (Figure 4). An overestimation of pollutant biodegradation using conventional Rayleigh-equation-streamline approach (eq 3) was expected when volatilization, advection and dispersion cause a reduction of pollutant concentration (*14*, *30*, *31*). The same effect can be considered for irreversible sorption. Dilution leads to displacement of pollutants out of a streamline but, in contrast to volatilization and irreversible sorption, does not remove contaminants out of the groundwater. Hence, although the portion of the pollutants leaving the streamline can be biodegraded after the transfer into another groundwater segment, the biodegradation of this portion is not ascertainable by the Rayleigh-equation-streamline approach. In a homogeneous aquifer where biogeochemical conditions are similar everywhere, a reliable extent of biodegradation can be obtained from the conventional Rayleigh-equation-based approach (eq 3) for a streamline as its vicinity may exhibit similar biodegradation rates. In many cases, however, aquifers are heterogeneous systems with strongly changing biogeochemical conditions. To improve the assessment of pollutant biodegradation based on the Rayleigh-equation-streamline approach, the determination of changes in isotope signatures for cross-sections along the pollutant plume is necessary. The obtained isotope data can be used to calculate pollutant biodegradation for a number of streamlines. Using these results, biodegradation for these cross-sections can be assessed using an appropriate conceptual model. Thus, further studies need to focus on two- or three-dimensional investigations to determine the influence of nonisotope-fractionating processes on the calculation of pollutant biodegradation using stable isotope fractionation analysis.

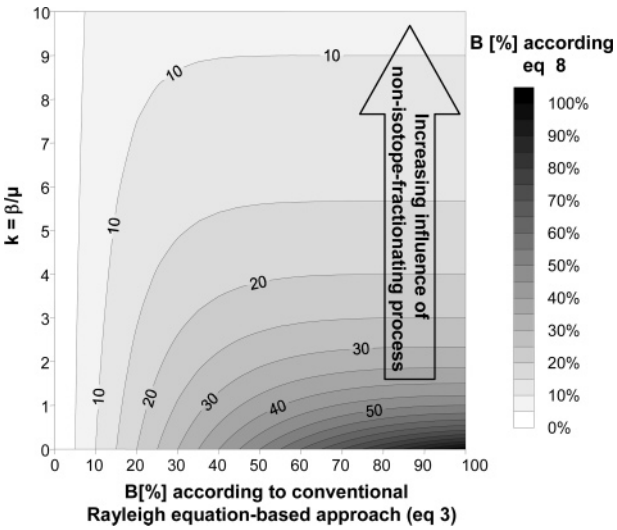

FIGURE 4. Influence of a nonisotope-fractionating abiotic process on the quantification of the percentage of biodegradation (*B*[%]) via the Rayleigh-equation-streamline approach. The *x*-axis gives the percentage of biodegradation according to the conventional Rayleigh equation approach (eq 3). The *y*-axis represents *k* which is the ratio between the rate constants of the nonisotope-fractionating abiotic process (β) and biodegradation (μ) ($k = \beta/\mu$). High values for *k* indicate a high influence of the nonisotope-fractionating abiotic process, and $k = 0$ represents systems with only biodegradation taking place. The black−white contours stand for the percentage of biodegradation calculated by the Rayleigh equation approach including the influence of a nonisotope-fractionating abiotic process (eq 8). For example, if the Rayleigh equation approach (eq 3) predicts *B*[%] to be 70%, the real *B*[%] for a value of $k = 1$ (the nonisotope-fractionating processes and biodegradation contributing equally to concentration decrease) would be only approximately 45% (according to eq 8). If the nonisotope-fractionating abiotic process becomes more dominant *B*[%] would be even lower, e.g., approximately 25% for $k = 3$.

Implication for the Application of the Stable Isotope Fractionation Analysis at Field Sites. In many cases there is insufficient information about biogeochemical conditions in a contaminated aquifer. As shown in this study, the combined analysis of carbon and hydrogen isotope fractionation can be used to determine whether the benzene biodegradation occurred under anoxic or oxic conditions. This concept has been applied previously to investigate under which biogeochemical conditions MTBE was biodegraded (*10*, *13*). However, analysis of carbon and hydrogen isotope fractionation may not only be used to distinguish between

biodegradation under anoxic or oxic conditions but also for the identification of predominant biodegradation pathways taking place in an aquifer (*32*). The basic requirement is that the isotope fractionation for at least carbon or hydrogen differs significantly for the various degradation pathways.

Stable isotope fractionation analysis can also be used to quantify biodegradation in a contaminated aquifer. The quantification of percentage of contaminant biodegradation using the Rayleigh-equation-based approach is quite robust with respect to aquifer heterogeneities along a flow path, including effects due to longitudinal dispersion, variable degrees of biodegradation, plume geometries and travel times (*15*). However, the conventional Rayleigh-equation-streamline approach (eq 3) can lead to an overestimation of biodegradation if dilution, volatilization or irreversible sorption affects pollutant concentration along a streamline between contaminant source and sampling well. To verify the influence of nonisotope-fractionating abiotic processes on the decrease in pollutant concentration, biodegradation can be calculated on the basis of the assumption that the biodegradation occurs before a nonisotope-fractionating abiotic process (scenario 1, eq 3). Alternatively, biodegradation can be quantified assuming that a nonisotope-fractionating abiotic process occurs before biodegradation (scenario 2, eq 4). If the results of both scenarios are similar, then biodegradation can be regarded as the predominant attenuation process. If the results of both scenarios differ significantly, then without knowledge about the influence of nonisotope-fractionating abiotic processes, the Rayleigh-equation-streamline approach can lead either to a significant overestimation or underestimation of biodegradation depending on the scenario assumed.

In several contaminated aquifers, measured concentrations (C_x) of toluene and xylenes were quite similar to concentrations predicted by the Rayleigh-equation-streamline approach (CB_x), indicating that biodegradation was the predominant attenuation process for these compounds (*7*, *9*, *12*). In other studies, it was shown that biodegradation of toluene and xylenes calculated by the Rayleigh-equation-streamline approach were similar to the results of independent methods (*29*, *33*). In contrast to these findings, measured benzene and MTBE concentrations (C_x) differed significantly from the concentrations predicted by the Rayleigh-equation-streamline approach (CB_x) (*9*, *13*), which may imply that nonisotope-fractionating processes such as dilution lead to a strong decrease in pollutant concentrations. Toluene and xylenes are known to be more easily biodegradable than benzene and MTBE under anoxic conditions (*4*, *28*, *34*). In general, the assessment of biodegradation by the Rayleigh-equation-streamline approach should lead to more reliable results for more biodegradable pollutants, which tend to form smaller contaminant plumes with a lower influence of dilution compared to more persistent pollutants.

Acknowledgments

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Supporting Information Available

History and hydrogeological conditions of the field site, map of the Zeitz field site, sampling, analytical methods, detailed derivations of equations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Applicability of stable isotope fractionation analysis for the characterization of benzene biodegradation in a BTEX-contaminated aquifer

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Field site

The investigated field site is located in the area of a former hydrogenation plant close to the city of Zeitz (Saxony-Anhalt, Germany). The hydrogenation plant was founded in 1938 to produce gasoline and lubricants. At the end of the Second World War, the plant was largely destroyed, leading to the entry of large volumes of raw materials as well as industrial goods into the subsurface. During the existence of the former German Democratic Republic (GDR), parts of the production facilities were reconstructed and later expanded with new plant sectors. Benzene production at the test site lasted from 1963 to 1990. After the re-unification of Germany in 1990 the plant was shut down because the production was no longer economically feasible. Consequently, all facilities were demolished and removed (*1,2*).

At the site two main heterogeneous aquifers can be distinguished, separated by a discontinuous lignite-clay layer of Tertiary age. The upper aquifer is composed of a 2 to 10 m thick layer of Quaternary and Tertiary sand and gravel deposits. The lower aquifer, with a thickness of 12 to 20 m, consists of sand and gravel deposited by an Eocene river. The general groundwater flow direction is to the north-northeast. The two aquifers have an organic carbon content of approximately 0.1 % (f_{oc} = 0.001) and a mean effective porosity of approximately 0.22. The water table varies between 8 and 10 m below ground surface (bgs) depending on the seasonal fluctuation over the year and the location (*1,2*).

In the near-source areas of the upper aquifer, the BTEX concentration can exceed 1000 mg/L. The dominant contaminants are benzene and toluene with concentrations up to 950 mg/L and 50 mg/L, respectively. Highest benzene concentration detected exceeding 1000 mg/L indicating non aqueous liquid benzene phases in the source area. Ethylbenzene and xylenes are only present in minor concentrations below 3 mg/L. In the lower aquifer, the BTEX concentrations are found up to 150 mg/L and mainly consist of benzene. At the test site, the predominant electron acceptor is sulphate (*3,4*). In addition, methanogenesis leads to minor microbial BTEX transformation in the contaminant plume but is more significant in the source area (*3*). Other electron acceptors such as oxygen, nitrate and ferrous iron play only a marginal role in biodegradation processes at the site (*3,4*). For hydrochemical and

isotopic analyses, groundwater samples were taken at several multi-level sampling wells (A, B, C, D, E) along a transect in the upper aquifer extending from a source area to the fringe of the contaminant plume following the main groundwater flow direction (Fig. 1).

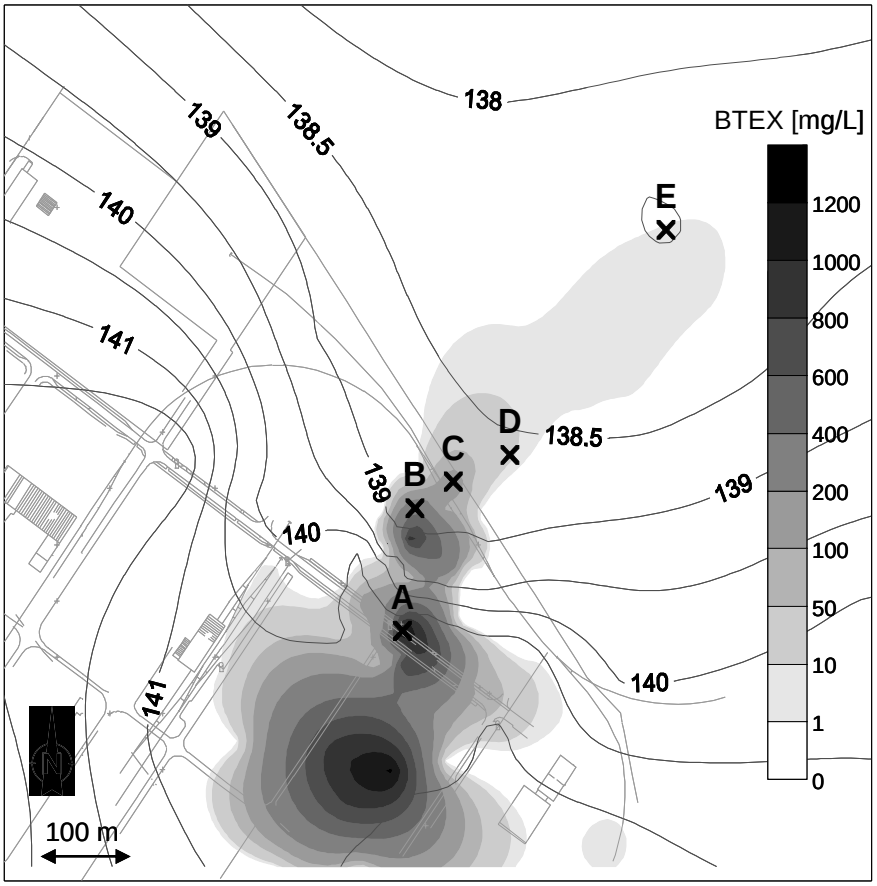

Figure 1. Map of the Zeitz field site showing the BTEX plume within the upper aquifer with the sampling wells of the investigated aquifer transect and groundwater table contour lines.

Chemicals

The chemicals were obtained in reagent quality from Merck (Germany) unless otherwise stated.

Sampling

For hydrochemical and isotopic analyses, groundwater samples were taken along a transect in the upper aquifer extending from the source area to the fringe of the contaminant plume along the main groundwater flow direction. Groundwater samples from the source area were collected by using submersible electrical pumps (MP1, Grundfos, Denmark) according to good sampling practice (*5*). The other wells were equipped with a multilevel packer system for vertical sampling of the contaminant plume (*6*). Small gas-driven double-valve-pumps (IMW-Innovative Measurement Technology Weiss, Germany) installed every 0.7 to 1.4 m were used with pumping rates up to 0.05 L/min to collect groundwater samples.

For the analysis of benzene isotope signatures, groundwater was filled into 1 L Duran® glass bottles sealed with Teflon®-coated caps (Schott, Germany) without headspace, thus avoiding evaporation. The water samples were adjusted to pH 10 to 12 using sodium hydroxide pellets to inhibit microbial activity. Water samples for BTEX concentrations analyses were collected in 100 mL brown glass flasks without headspace and sealed with glass stoppers. The samples were conserved by 1 mL sulphuric acid. For the analysis of sulphate and nitrate concentrations, groundwater was filled into 20 mL polyethylene bottles. To determine the iron concentration in groundwater, samples were collected in 20 mL polyethylene bottles. For conservation of the samples, the groundwater was adjusted to pH < 2 using nitric acid. Water samples for hydrogen carbonate analysis were collected in 250 mL polyethylene bottles. For the analysis of methane concentrations, 20 mL headspace vials were filled with groundwater and sealed with gas-tight caps. All samples were stored in the dark and at 4 °C.

Analytical Methods

For the analysis of carbon and hydrogen isotope signatures of benzene, the groundwater samples were extracted with 1 or 2 mL *n*-pentane, depending on benzene concentration. After shaking the bottles at least for 4 h at 20 °C the samples were cooled down to 4 °C in order to avoid evaporation effects during subsequent removal of the *n*-pentane phase which

in each case was filled into a 5 mL glass vial and sealed with a Teflon®-coated cap. Solvent extraction is considered not to affect the isotope signature of BTEX (*7*). To avoid evaporation effects the vials were stored on their heads at 4 °C until the analysis. The carbon and hydrogen isotope signatures of benzene were determined using two independent gas chromatography isotope ratio mass spectrometry (GC IRMS) systems. Each system consisted of a HP 6890 Series gas chromatograph (Agilent Technology, USA) into which aliquots of the *n*-pentane extract was injected via an AS Combi Pal auto sampler (Chromtech, Germany). The injector temperature was set at 250 °C. Benzene was separated from other groundwater compounds by a ZB-1 capillary column (60 m x 0.32 mm ID x 1 µm FD; Phenomenex, Inc. Torrance, USA). The temperature program started at 40 °C, was held for 5 min isothermally, was increased at a rate of 3 °C/min to 90 °C where it was held for 2 min, then was increased at 20 °C/min to 300 °C. Helium was used as carrier gas with a flow rate of 2.0 mL/min for carbon isotope ratio analysis and 1.4 mL/min for hydrogen isotope ratio analysis. After the separation, the benzene was transferred to a combustion furnace (MAT GC-III, Thermo Electron, Germany) for carbon isotope ratio analysis where it was converted at 980 °C on a CuO/Ni-catalyst to carbon dioxide and water. The water was separated *via* a Nafion® membrane (50 cm long, T = 0 °C, DuPont, USA). For the hydrogen isotope ratio analysis, the benzene was transferred to a pyrolysis device (GC-pyrolysis, Thermo Electron, Germany) consisting of a ceramic tube where the benzene was converted at 1400 °C to molecular hydrogen and graphite. The pyrolysis device was oxidized daily to remove the graphite remaining in the ceramic tube. After the conversion of the benzene, carbon dioxide and hydrogen were transferred on-line to a MAT 252 and 253 mass spectrometer (Thermo Electron, Germany), respectively, in order to determine the $^{13}CO_2/^{12}CO_2$ and $^{2}H^{1}H/^{1}H^{1}H$ ratios which were representative for the analogue benzene. Because the formation of H_3^+ ions contributes to the $^{2}H^{1}H$ signal, it was adjusted by a correction factor. The GC IRMS systems were calibrated using gases (CO_2 and H_2) with known isotope composition. The isotope composition of gases were determined by means of International Atomic Energy Agency (IAEA) reference materials (*8*) using elemental analysis.

The carbon and hydrogen isotope ratios were expressed in the delta notation ($\delta^{13}C$ and $\delta^{2}H$) in per mil [‰] units according to Equation 1. All samples were measured in at least 3 replicates. The reproducibility of $\delta^{13}C$ values and $\delta^{2}H$ values was always smaller than 0.5 ‰ and 10 ‰, respectively.

$$\delta^{13}C_{sample} \quad \text{or} \quad \delta^{2}H_{sample}\ [‰] = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) \cdot 1000 \qquad (1)$$

In Equation 1, R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$-ratios or $^{2}H/^{1}H$-ratios of the sample and an international standard, respectively. Vienna Pee Dee Belemnite (VPDB) was used as the standard for the analysis of carbon isotope signature and Vienna Standard Mean Ocean Water (VSMOW) was used as the standard for the detection of hydrogen isotope ratios (*8*).

Concentrations of benzene were determined with a gas chromatograph (Perkin-Elmer, USA) equipped with a flame ionization detector. The samples were injected via a headspace auto-sampler (HS 40, Perkin-Elmer, USA). Headspace sample vials were kept at 80 °C for at least 40 min. The headspace samples were transferred into the injector held at 169 °C in split-mode. Benzene was separated from other groundwater compounds by a capillary column (DBVRX, 75 m x 0.45 mm ID x 2.5 µm FD, Restek, Germany). The temperature program started at 33 °C and was held for 5 min isothermally, then increased at 6 °C/min to 200 °C and at 12 °C/min to 240 °C where it remained for 5 min isothermally. Helium was used as the carrier gas. The detection limit of the GC analysis was 2 µg/L for benzene. The relative error of benzene concentration analyses was smaller than 10 %.

Concentrations of dissolved oxygen, temperature, pH, redox potential, and electrical conductivity were determined immediately at the site by using the appropriate electrodes (Oxi 325, LF 325, pH 330, and pH 196T, WTW, Germany).

Ion chromatography was used to detect sulphate and nitrate concentrations. The ion chromatograph (DX 500, Dionex, USA) was equipped with an auto-sampler (AS 3500, Dionex, USA), a gradient pump (GP 40, Dionex, USA) and a conductivity detector (CD20,

Dionex, USA). The analytes were separated using an anion exchange guard column (IonPac AG11, 4 mm x 50 mm, Dionex, USA) in series with the analytical column (Ion Pac AS11, 4 mm x 250 mm, Dionex, USA) and a H_2O/NaOH gradient as eluent. Where necessary, samples were diluted to the appropriate concentration with deionized water. The detection limit for sulphate was 1.1 mg/L and for nitrate 0.28 mg/L.

Iron concentrations were detected via inductive coupled plasma-atomic emission spectroscopy (ICP-AES). Samples were injected via a pneumatic atomizer into the inductive coupled plasma of the ICP-AES system (CIROS^{CCD}, Spectro Analytical Instruments, USA) which had a temperature of 6000 to 8000 °C. At these temperatures, the elements in the sample are ionized leading to the release of radiation with an element-specific wavelength. Since the intensity of the radiation at these wavelengths is proportional to the concentration of the respective element, quantitative information could be obtained about the amount of iron in the groundwater sample. The detection limit for iron was 0.05 mg/L.

Hydrogen carbonate concentrations were analyzed via titration with hydrochloric acid to pH 4.3. The pH values were controlled by a pH-electrode (WTW, Weilheim, Germany). At the transition point of pH 4.3 the amount of hydrochloric acid added was used to calculate the hydrogen carbonate concentrations. The detection limit for hydrogen carbonate concentration was 0.1 mg/L.

Methane concentrations were determined by means of a GC system (GC-System XL, Perkin Elmer, USA) equipped with a thermal conductivity detector. The samples were transferred via an auto-sampler into the injector of the GC system at a temperature of 145 °C. The analytes were separated by a Poraplot-Q-HT column (30 m x 0,53 mm ID, Chrompack, Netherlands) adjusted at 20 °C isothermally. Helium was used as the carrier gas. The detection limit for methane concentration was 0.2 mg/L.

Rayleigh equation-based approach for quantifying pollutant biodegradation

For the description of isotope fractionation of biochemical reactions the Rayleigh equation can be used (*9*):

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \quad (2)$$

where R_t and R_0 are the stable isotope ratios of a compound at a given point in time and at the beginning of a transformation reaction, respectively. C_t and C_0 are the concentrations of a compound at the given point in time and at the beginning of the transformation reaction, respectively. The enrichment factor (ε [‰]) provides the link between the changes in stable isotope ratios (R_t/R_0) and the changes in the concentrations (C_t/C_0).

For the quantification of benzene biodegradation, three scenarios were considered: 1) biodegradation occurring before a non-isotope-fractionating process takes place, 2) a non-isotope-fractionating process occurring first, followed by biodegradation, and 3) both processes taking place simultaneously along a groundwater flow path between contaminant source and a sampling point.

For the first scenario, biodegradation of benzene (B [%]) can be quantified according to the conventional Rayleigh equation-streamline approach (*10*):

$$B[\%] = (1 - f_x) \cdot 100 = \left[1 - \left(\frac{R_x}{R_0}\right)^{\left(\frac{1000}{\varepsilon}\right)}\right] \cdot 100 \quad (3)$$

where R_x/R_0 is the change in isotope signature of the contaminant and f_x is the decrease in the pollutant concentration due to biodegradation along a flow path between a contaminant source and a sampling well ($f_x = CB_x/C_0$, where CB_x is the expected pollutant concentration at a sampling point resulting only from biodegradation), respectively. (Equation 3 is consistent with eq 3 in the main part of the publication.)

For the second scenario it was assumed that a non-isotope-fractionating process occurs first, after the end of which biodegradation begins. Assuming that at the transition from the non-isotope-fractionating process to biodegradation the pollutant concentration is given as $C_d = h_d * C_0$ (where h_d is the factor of decrease in pollutant concentration due to the non-isotope-fractionating process) and that the isotope ratio at the transition point is $R_d = R_0$, the Rayleigh equation can be used to calculate changes in pollutant concentrations caused by biodegradation along a streamline:

$$\frac{C_x}{C_d} = \left(\frac{R_x}{R_d}\right)^{\frac{1000}{\varepsilon}} = \left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}} \tag{4}$$

The overall changes in pollutant concentrations ($F_x = C_x/C_0$) along a flow path between a contaminant source and a sampling well can be expressed as:

$$F_x = \frac{\left(\left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right) \cdot C_d}{C_0} = \frac{\left(\left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right) \cdot h_d \cdot C_0}{C_0} = \left(\left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right) \cdot h_d \tag{5}$$

The percentage of biodegradation (B [%]) is then given as:

$$B[\%] = \left(\frac{C_d - C_x}{C_0}\right) \cdot 100 = \left(\frac{h_d \cdot C_0 - h_d \cdot \left(\left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right) \cdot C_0}{C_0}\right) \cdot 100 = \left(h_d \cdot \left(1 - \left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right)\right) \cdot 100 \tag{6}$$

or

$$B[\%] = \left(\frac{F_x}{\left(\frac{R_x}{R_0}\right)^{\left(\frac{1000}{\varepsilon}\right)}} \cdot \left(1 - \left(\frac{R_x}{R_0}\right)^{\left(\frac{1000}{\varepsilon}\right)} \right) \right) \cdot 100 \qquad (7)$$

(Equation 7 is consistent with eq 4 in the main part of the publication.)

In third scenario, biodegradation and a non-isotope-fractionating process occur simultaneously along a flow path between a contaminant source and a sampling well. Assuming that both processes follow first order kinetics, rates of biodegradation and a non-isotope-fractionating process (r_b and r_n, respectively) are given by:

$$r_b = \mu \cdot C \qquad (8)$$

and

$$r_n = \beta \cdot C \qquad (9)$$

with C as concentration of a pollutant, μ and β as rate constants of biodegradation and a non-isotope-fractionating process, respectively. Additionally, a constant transport velocity (v) was assumed which allows pollutant concentration resulting from the influence of biodegradation and a non-isotope-fractionating process over the distance from the source to a sampling point (x) to be expressed as follows:

$$C_x = C_0 \cdot \exp\left(-[\mu + \beta] \cdot \frac{x}{v} \right). \qquad (10)$$

The overall changes in pollutant concentrations along the flow path between source and a sampling point can be derived from Equation 10:

$$F_x = \exp\left(-[\mu+\beta]\cdot\frac{x}{v}\right). \quad (11)$$

The carbon isotope ratio of a pollutant at a sampling point can be described by the following expression:

$$R_x = \frac{^{13}C_x}{^{12}C_x} = \frac{^{13}C_0}{^{12}C_0}\cdot\exp\left(-[\mu_{13}+\beta_{13}]\cdot\frac{x}{v}+[\mu_{12}+\beta_{12}]\cdot\frac{x}{v}\right) \quad (12)$$

where ^{13}C and ^{12}C are the concentrations of carbon with mass 13 and 12 for a pollutant. μ_{12} and β_{12} are the rate constants of biodegradation and a non-isotope-fractionating process for carbon with mass 12. μ_{13} and β_{13} are the rate constants of biodegradation and a non-isotope-fractionating process for carbon with mass 13. Since kinetic isotope fractionation factors for biodegradation can be determined by rate constants ($\alpha = \mu_{13}/\mu_{12}$) (*9*), Equation 12 can be rearranged to:

$$R_x = R_0\cdot\exp\left(-\mu_{12}\cdot\left[\frac{\varepsilon}{1000}\right]\cdot\frac{x}{v}\right)\approx R_0\cdot\exp\left(-\mu\cdot\left[\frac{\varepsilon}{1000}\right]\cdot\frac{x}{v}\right) = R_0\cdot\left[\exp\left(-\mu\cdot\frac{x}{v}\right)\right]^{\frac{\varepsilon}{1000}} \quad (13)$$

with the assumption that that ^{13}C << ^{12}C, which is justified for natural conditions (^{12}C approximately 99% of total C). This leads to:

$$\frac{R_x}{R_0} = \left[\exp\left(-\mu\cdot\frac{x}{v}\right)\right]^{\frac{\varepsilon}{1000}} \quad (14)$$

The percentage of biodegradation (B [%]) along the flow path between source and a sampling point is given by:

$$B[\%] = \frac{\int_0^{t_x} r_b d\tau}{C_0} \cdot 100 = \frac{\frac{1}{v} \cdot \int_0^{x} r_b d\zeta}{C_0} \cdot 100 = \frac{\frac{1}{v} \cdot \frac{\mu}{\mu+\beta} \cdot C_0 \cdot v \cdot \left(1 - \exp\left[-(\mu+\beta) \cdot \frac{x}{v}\right]\right)}{C_0} \cdot 100 \quad (15)$$

Using Equation 11, Equation 15 can be rearranged to:

$$B[\%] = \frac{\mu}{\mu+\beta} \cdot (1 - F_x) \cdot 100 \quad (16)$$

By inserting the rearranged Equation 10

$$\mu + \beta = -\frac{v}{x} \cdot \ln(F_x) \quad (18)$$

and rearranged Equation 14

$$\mu = -\frac{v}{x} \cdot \ln\left(\left(\frac{R_x}{R_0}\right)^{\left(\frac{1000}{\varepsilon}\right)}\right) \quad (19)$$

into Equation 16, finally the percentage of biodegradation (B [%]) can be calculated by:

$$B[\%] = \frac{\ln\left(\left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right)}{\ln(F_x)} \cdot (1 - F_x) \cdot 100 \quad (20)$$

(Equation 20 is consistent with eq 7 in the main part of the publication.)

Assuming that the rate parameters for a non-isotope-fractionating process and biodegradation follow a constant relation k = β/μ, and using Equations 10 and 14, the influence of the non-isotope-fractionating process for the calculation of B[%] can be described by transforming Equation 16 to:

$$B[\%] = \frac{1}{1+k} \cdot \left(1 - \exp\left(-\mu \cdot \frac{x}{v} \cdot (1+k)\right)\right) = \frac{1}{1+k} \cdot \left(1 - \left(\left(\frac{R_x}{R_0}\right)^{\frac{1000}{\varepsilon}}\right)^{1+k}\right) \cdot 100 \qquad (21)$$

(Equation 21 is consistent with eq 8 in the main part of the publication.)

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